FUNCTIONAL SIGNIFICANCE OF SYMPATHETIC FIBER INGROWTH

IN THE HABENULA

DISSERTATION

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The physiological significance of noradrenergic sympathohabenular ingrowth following medial septal lesions was investigated. Following septal lesions, sympathetic fibers originating in the superior cervical ganglia are known to sprout into the medial habenular nuclei, and into the hippocampal formation. Previous work involving sympathohippocampal ingrowth showed that firing rates in septal animals with no ingrowth were higher than rates of septal animals with ingrowth and controls. Those results suggested that sympathetic ingrowth in the hippocampus had some functional capability in a modulatory manner. Thus, it was expected that noradrenergic habenular input of sympathetic origin would act as a neuromodulator, controlling firing rates of medial habenular neurons.

The primary aim of the present study was to determine if the peripheral sympathetic ingrowth into the medial habenular nuclei following a septal lesion is functionally significant. The study utilized the septal lesion induced sympathohabenular enrichment and superior cervical ganglionectomy to vary the degree of sympathohabenular innervation. Spontaneous activity of medial habenular neurons was recorded in six groups of rats anesthetized with chloral hydrate: controls (N=7), medial septal

lesions (N=13), septal lesion + bilateral ganglionectomy (N=8), sham + 6-OHDA (6-hydroxydopamine) (N=4), septal lesion + 6-OHDA (N=4), and septal lesion + bilateral ganglionectomy + 6-OHDA (N=6). The intraventricular injections of 6-OHDA were used to limit the noradrenergic innervation from both central and peripheral sources. Recording was done 4-7 weeks post-surgery and responses were analyzed by computer. All lesions, recording and injection sites, and ingrowth were verified histologically.

The results showed that firing rates of neurons of the medial habenulae in animals receiving septal lesions were significantly higher than rates of control animals and septal lesioned + ganglionectomized animals. Animals with 6-OHDA injections + septal lesions showed decreased firing rates when compared with higher rates of septal animals. Animals with septal lesions, bilateral ganglionectomies + 6-OHDA injections (given to remove noradrenergic central input) also had baseline firing rates rates that were significantly lower than rates of septal animals. These results indicate that the enhanced noradrenergic innervation in the medial habenular nuclei produces higher baseline firing rates, and when the innervation is removed (be it of central or peripheral origin), the baseline firing rates were lower.

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CHAPTER I

INTRODUCTION

General Background

During the past ten years, a large body of evidence
has accumulated to suggest that neuronal rearrangements
underlie the recovery of function phenomenon after brain
damage (6,8,9,23). It is established that neurons sprout
to form new synaptic connections in response to injury of
nearby and remote fibers in regions of the brain and spinal
cord (3,8,11,24). It is generally believed that alterations
in the denervated tissue initiate sprouting in responsive
neurons, but it is also recognized that changes in nerve
impulse flow to the neurons may initiate sprouting
(10,12,14). Despite the large amount of data related to
neuronal sprouting, the factors which regulate the
formation of new neuronal connections are not
completely understood. For example, a peculiar, but
specific form of neuronal sprouting occurs in the rat
brain. Peripheral noradrenergic fibers sprout specifically
in response to destruction of central cholinergic fibers
after septal lesions (9,12,43). This type of sprouting in
the central nervous system of the rat is one in which an
identified transmitter system (in this case, noradrenergic)

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sprouts in response to the damage of another specific transmitter system (cholinergic) (8,9,11).

Normally, peripheral sympathetic axons are confined to extracerebral blood vessels, but after lesions of the medial septal nuclei, they grow into the medial habenulae. This phenomenon is quite similar to the appearance of sympathetic fibers in the rat hippocampal formation after septal lesions (2,6,9,44). Septal ablation results in the sprouting of sympathetic terminals into the hippocampal formation from their normal innervation of the vasculature. Lesions of other areas, such as the anterior hippocampal formation and the fornix/fimbria also result in the appearance of sympathetic fibers in the dentate and hippocampal gyri (6,21,43,60). The nature of the septal projection to the habenulae and hippocampal formation is cholinergic, and suggests that after cholinergic denervation, sympathetic fibers may also appear in other brain regions.

The peripheral sympathetic noradrenergic fibers originate in the superior cervical ganglion, and will innervate non-neuronal sites in the central nervous system such as the cerebral vasculature, choroid plexus and pineal gland (3,15,53). The medial habenular nucleus is the only neuronal structure reported to be innervated by these sympathetic fibers (3). The functional significance of this particular innervation has not been investigated previously and stimulated interest to determine if noradrenergic

sympathohabenular ingrowth is of a functional nature.

The answer to this question is considered important because it may be used to provide evidence of the functional role of peripheral innervation of a central nervous system target. The hypertrophy of this sympathohabenular innervation following septal lesions is thought to have implications for recovery of function following brain damage (6,23,30).

The habenular complex receives inputs of both extra-pyramidal (in particular, striatal) and limbic origin, and is one of the few brain regions in which these two major systems are known to converge (34,49). The habenulae receive well-defined afferents from multiple sources which can be selectively manipulated. Therefore, this region is a useful model for the study of the phenomenon known as neuronal sprouting. As mentioned earlier, the growth of intact axon terminals in certain regions of the central nervous system following partial or complete deafferen-tation may play an important role in the recovery of function that is lost as a result of a lesion. Since adrenergic neurons possess a marked sprouting capacity, the habenular complex is one area in the adult rat brain that is regarded as useful for the study of adrenergic sprouting (24). The dissertation will specifically study the effect of deafferentation on the sprouting phenomenon by recording spontaneous firing rates in the medial habenular neurons after septal lesions. The

sprouting fibers are collaterals of neurons whose cell
bodies arise in the superior cervical ganglion, a structure
that can be deafferented without affecting other inputs to
the medial habenular nuclei.

Anatomy of the Habenular Complex

It is pertinent to include a detailed discussion of
the anatomy of the habenular complex to facilitate
understanding of the neuronal rearrangements that occur in
this area.

Dorsal Diencephalic Conduction System

The afferent and efferent connections of the habenular
complex describe the anatomy of the dorsal diencephalic
conduction system (61). This system provides for the
conduction of information from the limbic forebrain to the
limbic midbrain areas (61). Actually, there are two primary
pathways connecting the limbic forebrain with the midbrain,
the principal one being the medial forebrain bundle. This is
a bundle of fibers that receives noradrenergic axons from the
locus coeruleus, which is a significant area containing cell
bodies of origin for noradrenergic neurons. The medial
forebrain bundle then courses anteriorly to innervate the
entire cerebral cortex and hippocampus (57). The other
route, the dorsal diencephalic conduction system, passes
through the epithalamus, and originates in the anterior
portion of the medial forebrain bundle, traveling
primarily in the stria medullaris to terminate in the

habenular complex (61). The stria medullaris can be described as a narrow, compact fiber bundle that extends along the roof of the third ventricle to the thalamus on each side, and terminates posteriorly in the habenular nuclei. It is composed of fibers originating in the septal area, the lateral preoptic nucleus, and the medial segment of the globus pallidus (34,49,61).

The stria medullaris, the habenular complex, and a third structure, the habenulo-interpeduncular tract (the fasciculus retroflexus of Meynert) comprise the dorsal diencephalic conduction system. The habenulo-interpeduncular tract is made up mostly of efferent fibers from the habenular complex to reach the midbrain (61). This conduction system provides an opportunity for interaction of activity in motivational systems with movement systems in the striatum and midbrain. In other words, it provides an alternative to the descending medial forebrain bundle for the conduction of information from the limbic forebrain to limbic midbrain areas. In addition, this path contains converging limbic and striatal efferents within the lateral habenular nucleus. Considering that the limbic system is believed to control behaviors associated with emotions, the neuroanatomical convergence mentioned earlier between the limbic and the striatal systems could possibly regulate the control of movements by motivational processes (49,61).

The habenular complex is composed of two separate

nuclei, medial and lateral, in addition to a habenular commissure. The habenular commissure is not actually thought of as such, but instead as a decussation of fibers traveling in the stria medullaris. Most of the known fibers of the dorsal conduction route terminate in, or originate from one of the two sets of nuclei, and therefore, the anatomical organization of this route is described in terms of afferents to, and efferents from the habenular nuclei (34,61). Since the dissertation deals with the medial habenular nuclei, its afferents and efferents will be discussed first. The afferents and efferents of the lateral habenulae will then be discussed to provide a complete description of the habenular complex.

Anatomically, the medial habenular nucleus is a bilateral, symmetrical pair of nuclei located along the caudal half of the dorsomedial edge of the thalamus (67). Medially, the medial habenular nucleus is lined with ependyma, or cellular membrane facing the dorsal part of the third ventricle. Laterally is the lateral habenular nucleus. The fiber bundles of the stria medullaris approach the medial habenular nucleus from its rostro-lateral aspect, and cover the dorsal pole of the nucleus. Caudally, the nucleus stretches into the beginning of the habenular commissure. At the ventro-caudal pole, the habenular efferent fibers turn ventrally into the habenulo-interpeduncular tract (67).

Afferents of the Medial Habenular Nucleus

Afferents of a cholinergic nature.--The medial
habenular nucleus receives most of its afferents through
the stria medullaris, and it has been shown that a major
portion of this pathway is composed of collaterals of
medial forebrain bundle fibers. Anterograde degeneration
studies and horseradish peroxidase methods have shown that
the septal area, in particular the postcommissural septum,
is the main source of afferents to the medial habenular
nucleus, and further, that these septo-habenular fibers
travel in the stria medullaris (11,24,34,49,61). A
significant number of stria medullaris fibers originating
from the septum pass through the habenulae, without
terminating, on their way to the midbrain tegmentum (61).
Specifically, the rostral part of the medial habenular
nucleus receives afferents from the septo-fimbrial nucleus,
and the more caudal areas receive fibers from the nucleus
triangularis septi (34,61). These two septal areas can be
described as the postcommissural septum, whereas the area
known as the precommissural septum appears to contribute
very little to the innervation of the medial habenulae
(49). Initially, the septo-habenular fibers pass in the
descending columns of fornix, later joining with the stria
medullaris to occupy the most medial position.

The diagonal band of Broca (a white fiber bundle
descending in the precommissural septum) also sends a

small, but significant number of connections to the medial

habenular nuclei (34,49,61). This projection has been

found to contain many neurons which stain strongly for

acetylcholinesterase (AChE), a primary indication that a

cholinergic projection from the septal-diagonal band area

to the habenulae exists (49). In addition, choline

acetyltransferase (ChAT), a cholinergic neuronal marker,

was found to be differentially distributed in the habenulae,

with a five-fold increase in activity in the medial

nuclei (26). Much of this enzymatic activity was

attributed to cholinergic afferents contained within the

stria medullaris. It is known that the septal area gives

rise to habenular cholinergic afferents through this system,

and the nucleus of the diagonal band of Broca is contained

within the medial area of the septal region, which

possesses a strong cholinergic system (26,52). The medial

habenulae receive a strong cholinergic projection from the

septal area, meaning cholinergic synaptic terminals are

located within the nuclei. However, slice culture studies

confirm the presence of cholinergic neurons in the medial

habenulae (36). It has also been suggested that the

habenulo-interpeduncular pathway is cholinergic (19).

Noradrenergic and serotonergic afferents.--There

is evidence of a smaller number of connections to the

medial habenular nucleus from the median raphe, the

ventral central gray and the dorsolateral tegmental

nucleus (34,49,61). The projection from the median raphe probably arrives via the habenulo-interpeduncular tract, and gives rise to the serotonergic terminals found in the medial nucleus (61). By using the glyoxylic acid fluorescence method, it has been demonstrated that a minimal number of projections from the ventral central gray and the dorsolateral tegmental nucleus may be composed of noradrenergic fibers (60). These fibers travel to the habenulae by way of the dorsal bundle to the medial forebrain bundle, and then by way of the stria medullaris.

An important source of noradrenergic fibers of peripheral origin also reaches the medial habenular nucleus, arising from the superior cervical ganglion after septal lesions in the rat (4,11). There is some disagreement concerning the presence or absence of normal sympathohabenular innervation. Some researchers have demonstrated the presence of normal sympathetic innervation to the habenulae (3,4,65). Others found no evidence that such innervation exists in the normal animal, and demonstrate only sympathohabenular hypertrophy in response to cholinergic denervation (11).

In addition to the proposed noradrenergic fibers of central origin, afferent fibers from the brainstem monoamine cell groups travel to both medial and lateral nuclei (57,61). The cell bodies of origin for norepinephrine are contained in the locus coeruleus, and send axons through the dorsal bundle to course anteriorly through the medial forebrain

bundle to innervate the cerebral cortex and hippocampus (57).
This noradrenergic projection, as well as the projections
from the ventral central gray and dorsolateral tegmental
nucleus, is a sparse one (61).

An even smaller pathway from the neurons of the
midline ventral tegmental area projects to the medial
sector of the medial habenulae. Using a microradiochemical
assay for chatecholamines, it has recently been found that
this pathway is dopaminergic (51).

It was once believed that the habenular complex was
closely associated with the olfactory system, but when
olfactory tubercle or piriform cortex lesions were made to
study degenerating stria medullaris fibers, it was seen
that these fibers merely pass through the habenulae on
their way to termination in the mediodorsal nucleus of the
thalamus (61).

In summary, the largest source of central input to the
medial habenular nucleus is cholinergic, and descends from
the postcommissural septum, with less numerous ascending
monoamine afferents from the median raphe, ventral central
gray, and brainstem monoamine cell groups. These projections
provide the medial habenulae with their known cholinergic
and noradrenergic innervation (61).

Efferents of the Medial Habenular Nucleus

The medial habenular efferents comprise the central
portion of the fasciculus retroflexus, which is the

projection from the medial habenular nucleus to the inter-
peduncular nucleus, where it terminates (45,61,63). This
nucleus lies ventromedially in the mesencephalon, and is
considered to belong to the limbic midbrain area (63). Its
physiological role is connected with autonomic functions.
The interpeduncular nucleus in turn projects to hypothal-
amic, limbic forebrain, and brainstem areas (61).
This habenulo-interpeduncular projection is described as
being topographically organized, meaning that medial
regions of the medial habenular nuclei project to
ventral portions of the interpeduncular nucleus, and
lateral regions project to the dorsal interpeduncular
nucleus (7,45). The projections from the medial and
lateral habenulae to the interpeduncular nucleus were
confirmed by tracing anterogradely transported
horseradish peroxidase (45). A sparse projection to the
interpeduncular nucleus was traced from the horizontal
limbs of the nucleus of the diagonal band of Broca (45).
There is substantial evidence that the habenulo-
interpeduncular projection is, in part cholinergic, and
that some of its fibers also contain substance P
(45,63). Even though the interpeduncular nucleus makes
reciprocal connections with most of its afferent sources
(i.e., diagonal band nucleus), it does not appear to do so
with its most important source of afferent input, the
habenular complex (7).

Afferents of the Lateral Habenulae

The lateral habenulae receive the majority of afferents from the lateral preoptic hypothalamic areas, and the entopeduncular nucleus (34,49). An example of the research supporting the origin of the afferent projection is of an electrophysiological nature. Evoked potentials recorded in the lateral habenulae in response to lateral preoptic stimulation consisted of several components: one that was mediated by a direct pathway via the inferior thalamic peduncle and the stria medullaris, and the others mediated by a polysynaptic pathway (47). The entopeduncular nucleus is considered to be the internal segment of the globus pallidus of non-primate mammals, and also is thought to be a GABA-containing system (34,49).

Other afferents to the lateral habenulae are via the mesohabenular dopamine system. Fluorescence histochemistry, in conjunction with lesion studies, document the existence of this pathway by the identification of cell bodies in the ventral mesencephalon, whose axons ascend with the habenulo-interpeduncular tract to terminate in the lateral habenulae (40,51,59).

Efferents of the Lateral Habenulae

The efferents of the lateral habenulae are many, and thus somewhat confusing. The lateral habenulae has been shown to project to the hypothalamus, septum, ventral tegmental area, substantia nigra, and particularly to the

median and dorsal raphe nuclei (34,63). It has been suggested that either GABA or substance P is a likely transmitter candidate in lateral habenular efferents to the raphe nuclei (63). To further clarify this suggestion, the transmitters contained in the efferents of the lateral habenulae were examined after kainic acid injections into the habenular complex (63). Kainic acid was chosen because it destroys the habenular cells, but spares fibers of passage. The dorsal raphe nuclei receive afferents from forebrain structures and from the lateral habenulae, leading to a confusion of possible efferent transmitters. Therefore, the kainic acid injections were believed to circumvent the problem of interpretation. These injections produced no decrease in glutamic acid decarboxylase (GAD) in the dorsal raphe, but large depletions of substance P were found in that region, suggesting that the projection from the lateral habenulae to the dorsal raphe contains substance P (63).

To summarize, both medial and lateral nuclei receive fibers (in substantially different concentrations) from the basal forebrain regions, including the septum, nucleus basalis and the lateral preoptic nucleus. Based on horseradish peroxidase labeling studies, neurons projecting to the entire habenular complex are located in the magnocellular forebrain nuclei, including the substantia innominata (29,49). The lateral habenular nucleus receives an additional projection from the medial segment of the

globus pallidus, known in the rat as the entopeduncular

nucleus. Both nuclei project by way of the habenulo-

interpeduncular tract to the nucleus interpeduncularis

(especially the medial projection), and to a medial zone of

the midbrain tegmentum, meaning the lateral projection

travels further to the median and dorsal raphe nuclei.

Habenulo-Pineal Connections

The habenular nuclei are known to be in close

proximity to the pineal gland, which would facilitate

functional interaction between the two structures.

Evidence for a direct habenulo-pineal fiber pathway was

obtained by recording extracellular potentials in the

pineal gland activated by habenular stimulation (53). The

pineal gland is exclusively innervated by the peripheral

autonomic nervous system, and contains a dense network of

sympathetic (noradrenergic) fibers (3,65). Habenular

commissure fibers have also been shown to penetrate

into the proximal part of the pineal gland (3,53). The

adrenergic innervation is continuous with the nerve plexus

in the lamina intercalaris of the pineal, which is the name

given to the proximal part of the epiphyseal stalk, a

connective tissue structure containing nerve fibers and

parenchymal cells, (those that are specific to the pineal

gland) (3,65). This stalk attaches the pineal gland to the

epithalamic portion of the diencephalon. The superior

cervical ganglion contributes a bilateral sympathetic

nerve supply to the medial habenular nucleus (3,61,65).

This arrangement, considered with the apparent continuity

of the sympathetic innervation in the pineal gland and

lamina intercalaris with the medial habenular nucleus,

is suggestive of functional interconnections between

these structures (3). Cells of the pineal gland, stalk

and lamina intercalaris also contain serotonin (5-HT),

whose synthesis and content in the pineal follow

circadian rhythms, meaning they are related to biological

variations with an approximate twenty-four hour cycle (3).

These in turn, are under neural control by way of

sympathetic innervation originating in the superior

cervical ganglia (65).

Establishment of Cholinergic Pathways and Cell Bodies

It is necessary to definitively establish the

existence of cholinergic cell bodies within the medial

habenulae, as well as a cholinergic pathway traveling to

the medial habenulae, because it is only in response to

cholinergic denervation that sympathohabenular sprouting

occurs. Explant cultures from the rat habenulae have been

shown to contain cholinergic cell bodies, and to have

developed extensive, AChE-positive neurite outgrowth

(36,52). When activity for ChAT was tested, it was seen

to originate from fibers coming from the septal area, and

traveling through the stria medullaris (36). Using AChE

histochemistry and biochemical ChAT and AChE determinations,

it has been conclusively determined that ChAT and AChE

activity in the habenulae and hippocampus originate

primarily in axons from cholinergic neurons located in the

medial septum (24,25,52). Specifically, the cholinergic input

originates from cell bodies located in the diagonal band of

Broca and medial septum. Rimvall, et al. have shown

that fetal rat septal explants cultivated alone develop

ChAT activity, and that this development is increased by

co-cultivation with explants from natural targets of the

septal cholinergic neurons, such as from the hippocampus

and habenulae. It was concluded that this ingrowth of

septal cholinergic fibers into the hippocampus and habenule

is target-specific, since cerebellar explants cultured with

septum show neither an ingrowth of AChE-containing septal

fibers, nor an increase in ChAT activity (52). It is

interesting to note that habenular AChE positive fibers

were not found to grow into a co-cultivated hippocampal

explant (52).

The activity of ChAT was also examined in a long-term

study of embryonal medial habenular cultures, in which

extrinsic fiber systems would have degenerated over

time (36). A high activity of ChAT in the rat habenulae

was reported, which led to the suggestion that

cholinergic cell bodies are present in the habenular

nuclei. Cultures of adjacent thalamic tissue were used as

controls in this study, because thalamic tissue is devoid

of intrinsic cholinergic neurons. The control tissue

displayed ChAT activity that was 150-fold lower than that
displayed in the medial habenular nuclei (36).

Cholinergic projections from the septal-diagonal band
area to the habenular nuclei exist (24). The nucleus of
the diagonal band of Broca, which is considered to be part
of the septal region, projects to the habenular nuclei.
The medial area of the septal region is that area which
contains the nucleus of the diagonal band of Broca,
and is known to possess an impressive cholinergic
system. Therefore, it should be evident that the septal
area sends habenular cholinergic afferents via the stria
medullaris. After examining levels of ChAT in the lateral
and medial habenulae, Gottesfeld and Jacobowitz found ChAT
to possess activity five times greater in the medial
habenulae than in the lateral habenulae (26). A large
proportion of this enzymatic activity was attributed to
cholinergic afferents contained within the stria medullaris.
It was concluded that cholinergic cells of the nucleus of
the diagonal band of Broca are likely sources of
cholinergic projections to the habenular nuclei (26).

Ultrastructural Features of the Medial Habenulae

The ultrastructural features of the medial habenulae
relate to AChE histochemistry in an interesting way. There
are two kinds of neurons present in the medial habenulae,
those with a poorly developed rough endoplasmic reticulum
(rER), and those with a much better developed rER (62).

The latter were found to stain well for AChE, whereas the former had little or no AChE activity. The habenulae is considered to be the one area in which there exists an exception to the rule that all cholinergic neurons have high AChE levels (62).

All the neurons of the medial habenulae have three ultrastructural features in common: an irregularly indented nucleus, scattered Golgi complexes, and the occasional presence of nucleolus-like bodies in the cytoplasm (67). The histochemical differences relating to AChE can be localized in the habenular complex, meaning that the enzyme localized in the extracellular spaces comes from the synthetic activity of intrinsic neurons. This is supported by the fact that transection of the stria medullaris does not significantly affect AChE levels in the habenular complex (67). Since some of the afferents to the habenular complex are known to operate through specific transmitters such as acetylcholine and substance P, some researchers have speculated that ultrastructual and histochemical differences can be reliable markers for the identification of the two medial habenular neuronal types, as they relate to different neurotransmitter systems (62).

Evidence of Synaptic Plasticity

Now that the anatomy and histochemistry of the medial habenulae has been established, it is necessary to provide evidence that the habenular complex, in particular the

medial habenular nucleus, is a central site capable of synaptic plasticity and reinnervation. A composite study of synaptic reorganization in the rat medial habenular nucleus after stria medullaris transection was conducted by Zimmer, et al. (67). They found by quantitative electron microscopy that the normal medial habenular nucleus had a majority (84%) of synapses with asymmetrical thickenings, and were distributed mainly on dendritic spines. The rest of the synapses were described as having symmetrical thickenings and were distributed on dendritic shafts and cell bodies. Zimmer, et al. also found that some of the terminals making asymmetrical contacts had complex structures with infoldings of the plasma membrane. These terminals are believed to resemble the complex hippocampal mossy fiber terminals. When the stria medullaris was lesioned unilaterally, both asymmetrical and symmetrical synapse types degenerated (67). The hypothesis was examined that in the medial habenular nuclei, the denervated sites were reinnervated by the formation of new presynaptic terminals by elements already located in the denervated areas. Therefore, a few days after a lesion, degeneration is apparent, but the degenerating terminals are removed progressively at longer post-operative survival times. The numbers of non-degenerating terminals are low immediately after a lesion, but recover as degeneration is removed. This specific example was concerned with destruction of ipsilateral afferents, but because of evidence of

reinnervation, the authors felt there was a possibility that the contralateral fiber system of the stria medullaris moved into the vacated sites made available by the lesion. The point is the medial habenular nucleus is a central site capable of being reinnervated after deafferentation. It can be compared to other sites where reinnervation occurs because restoration of synapse numbers after partial de-afferentation has been described in other central nervous system sites, including the lateral septal nucleus, and stratum moleculare of the dentate gyrus (67).

Specifically relating to this dissertation, it has been shown in some studies that after a septal lesion, peripheral sympathetic fibers appear in the medial habenulae (11), while others indicate there is a normal sympatho-habenular innervation which hypertrophies after septal lesions (3,4,65). Another researcher indicates that normal sympathohabenular innervation is confined to the dorso-caudal aspect of the medial habenulae, but after cholinergic denervation, there is ingrowth into the entire medial habenulae (24). As the sympathohabenular hypertrophy is similar to the appearance of sympathetic fibers in the hippocampal formation after septal lesions, this re-arrangement is described as peripheral noradrenergic neurons replacing central cholinergic neurons (2,9,11,24,43). The decrease in cholinergic neurons is represented by a substantial decline in ChAT activity when either the septum or stria medullaris is lesioned (24,25).

Evidence of Noradrenergic Innervation After Septal Lesions

When the medial habenular nucleus was stained for noradrenergic histofluorescence, in the normal animal there was an occasional thin varicose fiber in some sections, which is the characteristic appearance of central noradrenergic neurons (11,42,43). Adjacent sections stained for AChE activity revealed strong staining around the medial habenular neurons, indicating that the cholinergic transmitter system is intact, as one would expect in a normal animal (2,11). Four weeks after a septal lesion, the medial habenulae revealed coarse, brightly fluorescent fibers, which were similar in appearance to peripheral sympathetic fibers. Their peripheral origin was strongly suggested by their complete absence after a bilateral superior cervical ganglionectomy (11).

It has been suggested that this noradrenergic innervation could partially emanate from central neurons (such as those from the monoamine cell groups), as well as from the superior cervical ganglia (SCG) of the peripheral sympathetic system (24,25). This rationale relates to the fact that after stria medullaris transection, noradrenergic axon terminals already present in the habenulae undergo collateral sprouting. In one study, there were three groups of animals which received bilateral stria medullaris (SM) lesions, then were followed a month later by either dorsal NA bundle lesions

(to remove central input), or SCG's (thus removing peripheral input) to examine the concentration of norepinephrine in discrete habenular regions (25). After a SM lesion, norepinephrine concentration increased, but this lesion-induced increase was significantly reduced in the medial habenulae by ganglionectomy. The lesions of the NA bundle also reduced this increase, but led to a reduction of norepinephrine in both medial and lateral nuclei. The uneven reduction in norepinephrine in discrete regions of the partially deafferented habenulae was associated with noradrenergic fibers coming from the SCG as well as from brainstem nuclei (25). The results of this study prompted interest to examine sympathohabenular ingrowth after septal lesions to see if it is strictly of peripheral origin, as suspected, or a combination of peripheral and central innervation.

Recovery of Function after Septal Lesions

The significance of sympathetic ingrowth into the hippocampal formation following a septal lesion has been related to recovery of function following brain damage (6,22,23,30,33,37). For example, rats with septal lesions were tested on the recovery of a spatial behavior, specifically chosen to assess recovery of function (30). A maze task was used because it is sensitive to hippocampal dysfunction, dependent on cholinergic systems, and recovery of performance for this task occurs after

medial septal lesions. The rate of recovery was enhanced in rats with septal lesions and simultaneous ganglionectomies. Based on the results, the authors suggested that sympathetic ingrowth retards recovery processes for maze performance (30). Behaviors considered to "recover", and closely associated with the growth of sympathetic fibers into the hippocampus after septal damage were tactile reactivity and open-field activity (5,6). As some behavioral deficits recover, this indicates that the behavior returns toward pre-lesion baselines, but recovery does not necessarily mean a complete cessation of aberrant behavior after a lesion. During the four-week period following septal ablation, some behavioral deficits recover as sympathohippocampal ingrowth increases concomitantly (5,6). Depending upon the behavioral paradigm being studied, this neuronal rearrangement either retards or facilitates recovery of function (6,30,37). Behaviorally, the septal lesion results in a syndrome that is composed of hyperreactivity, hyperemotionality, hypoactivity and hyperalgesia (6). Even though it has not been conclusively shown that the habenulae is in any way related to the septal syndrome, the importance of the behavioral discussion lies in its implications for neural mechanisms.

Reduced cholinergic activity due to medial septal ablation or atropine treatment reduces hippocampal glucose metabolism (although not permanently). This is a

condition that is associated with Alzheimer's Disease (31,32,33).

Previous Work Specifically Leading to the Dissertation

It has been shown that sympathohippocampal ingrowth is functional electrophysiologically (2). Rats with septal lesions (which will deplete centrally mediated hippocampal norepinephrine, in addition to destroying cholinergic cell bodies), and a bilateral superior cervical ganglionectomy, show no sympathetic ingrowth when the hippocampus is examined histochemically (2). Septal lesioned animals have brightly fluorescent fibers in the dentate gyrus of the hippocampus four weeks post-surgery (2). Central noradrenergic input to the hippocampus has been reported to act as a possible neuromodulator, serving to control and reduce spontaneously active baseline firing rates (66). Norepinephrine is often an "inhibitory neurotransmitter" in the central nervous system because Purkinje and cerebrocortical neurons are inhibited following stimulation of the locus coeruleus, or following iontophoretic application of norepinephrine (54,55,56,64,66).

More specifically, the iontophoretic application of norepinephrine (Ne) in the hippocampus was studied (54). It was found that the majority of cells tested were inhibited during and after the application of Ne. Cells in 6-hydroxydopamine (6-OHDA) treated rats fired faster than those in normal rats. Another study was

conducted in which the electrical activity of single pyramidal cells in the hippocampus was monitored extracellularly during electrical stimulation of the locus coeruleus in anesthetized rats (55). The stimulation of the locus coeruleus produced a long lasting inhibition of spontaneous activity of pyramidal cells, an inhibition that was absent in 6-OHDA treated rats. A third study dealt with the effects of locus coeruleus stimulation of evoked hippocampal unit activity (56). Most of the neuronal activity in the hippocampus in awake, freely moving rats was inhibited by electrical stimulation of the locus coeruleus. The cells were also inhibited by a loud auditory stimulus (tone), and the inhibitory responses to the tone and to locus coeruleus stimulation were antagonized by an intracisternal injection of 6-OHDA. Another study examined the interaction of iontophoretically applied Ne with the responsiveness of cortical neurons to acetylcholine (64). Norepinephrine differentially suppressed the spontaneous firing rate more than activity during acetylcholine-induced excitation, such that the excitatory response was enhanced relative to background discharge in most of the cells tested. These studies would indicate that in the absence of such input, baseline firing rates would be higher than normal when compared with control animals, or lesioned animals with ingrowth.

It was surmised that peripheral noradrenergic input to the hippocampus would also reduce spontaneous firing rates. In a study conducted four years ago to examine sympathohippocampal ingrowth, it was expected that baseline firing rates in the dentate gyrus of the hippocampus would be higher in the animals with septal lesions and bilateral ganglionectomies. This group of animals was compared with normal controls and animals with septal lesions. The findings corresponded well with expectations in that animals with septal lesions and bilateral ganglionectomies had higher baseline firing rates in the hippocampus than controls or septal-lesioned animals (2).

An initial study of the current sympathohabenular research was begun two years ago, but terminated with puzzling results. The septal lesion-induced sympathohabenular enrichment was utilized to vary the degree of sympathetic innervation. It was hypothesized that noradrenergic habenular input of sympathetic origin would act in an inhibitory fashion, controlling the firing rates of medial habenular neurons. The hypothesis was evaluated by measuring changes in spontaneous firing rates of medial habenular neurons when the sympathetic innervation was removed or enriched. The expectation was to find significantly elevated firing rates in the group of animals with medial septal lesions and bilateral ganglionectomies. There would be no peripheral noradrenergic input to the medial habenular region if the SCG were removed. Contrary

to expectations, the firing rates were found to be higher in the group of animals with medial septal lesions (with ingrowth) when compared with a control or a septal lesion + SCG group (no ingrowth).

One explanation would suggest that noradrenergic input to the medial habenulae is of a direct excitatory nature, rather than an inhibitory one. It was expected that firing rates of the septal lesioned group would be lower, due to the supposed inhibitory effects of norepinephrine on spontaneous firing rates. Another possible explanation for the previous results is that central noradrenergic afferents to the habenulae were not destroyed by the septal lesion. The central afferents might be exerting an inhibitory effect when the peripheral noradrenergic fibers innervated the medial habenulae. A possible inhibition of inhibitory central control would be in effect, and would serve to create an excitatory effect. An excitatory effect could also be produced if another central neurotransmitter system was present, such as a serotonergic one, also exerting an inhibitory effect.

Specific Aims of the Dissertation

The major aim of the dissertation was to determine if sympathohabenular ingrowth is of a functional nature. In view of the preliminary, unexplained findings, a secondary aim became apparent. It was considered necessary to repeat the previous study to determine if the same

results would be obtained, specifically to ascertain if septal-lesioned animals would produce higher baseline firing rates in the medial habenular nuclei than either controls or lesioned + ganglionectomized animals. The significance of sympathetic innervation of the habenulae following septal lesions has important implications for recovery of function following brain damage, since collateral sprouting may aid or retard recovery.

Both aims were evaluated by measuring changes in spontaneous firing rates of medial habenular neurons when both peripheral and central sympathetic innervation is manipulated. Fluorescent and AChE histochemistry was utilized to determine the presence of central and/or peripheral noradrenergic fibers in the medial habenular nuclei before and after a septal lesion, and to visualize cholinergic denervation after a septal lesion.

Injections of 6-hydroxydopamine (6-OHDA), a non-selective catecholaminergic neurotoxin, will be used as a tool to deplete either central or peripheral noradrenergic innervation in the medial habenulae.

CHAPTER II

METHODS

Experimental Groups

The subjects were fifty-nine male hooded rats of the
Long-Evans strain (300-400g) assigned to the following six
groups: (1) normal control (with central and possibly
peripheral innervation); (2) Sham-operated + 6-OHDA (cental
innervation depleted); (3) septal lesion (sympathohabenular
enriched, possibly from both peripheral and central origins);
(4) septal lesion + 6-OHDA (no peripheral or central
enrichment); (5) septal lesion + bilateral ganglionectomy
(no peripheral enrichment); (6) septal lesion + bilateral
ganglionectomy + 6-OHDA (again, no peripheral or central
enrichment). All surgical procedures were carried out at
the same time on each animal to minimize post-operative
trauma resulting from the combination of surgeries in the
experimental groups. Seven out of the original fifty-nine
animals died due to post-operative complications, before
the recording phase started. These complications were
mostly due to the 6-OHDA injections, and left the following
numbers of animals in each group for electrophysiological
and histological procedures: control - 8; sham + 6-OHDA -
6; medial septal - 14; medial septal + 6-OHDA - 7; medial
septal + bilateral ganglionectomy -9; medial septal +

bilateral ganglionectomy + 6-OHDA - 8.

Injections of 6-OHDA must be given intra-cerebroventricularly in order to cross the blood-brain barrier in adult rats. It is actively accumulated by presynaptic transport processes, and this reflects an actual degeneration of axons and terminals (35,39). If given peripherally, it causes depletion of norepinephrine from sympathetically innervated peripheral organs (39). Peripheral administration produces peripheral adrenergic sympathectomy, but central administration produces central catecholaminergic depletion. For example, intracerebral administration of the drug leads to a progressive disappearance of noradrenergic neurons in the locus coeruleus of the rat (18). The rate of disappearance is considered to be biphasic, with a rapid cell loss within the first forty-eight hours, followed by a further reduction in nerve cell bodies over a period of one month (18). The initial cell loss represents a direct involvement of noradrenergic perikarya, having taken up 6-OHDA to undergo immediate destruction. The subsequent progressive disappearance of neurons is thought to result from selective retrograde degeneration, after lesions of noradrenergic axons (18). Most norepinephrine nerve endings are permanently depleted after intraventricular 6-OHDA (18,39,46). It is established that noradrenergic nerve endings originating from the locus coeruleus or the superior cervical ganglion are situated in the medial

habenulae after septal lesions. As this area is also situated in the vicinity of the cerebral ventricles (the site of injection), both peripheral and central nerve endings would be expected to undergo immediate and permanent destruction after a 6-OHDA injection.

The use of the neurotoxin itself can be used as a tool to assess how 6-OHDA affects central versus peripheral fibers, depending upon its site of injection (18,39,46). The original rationale for using this neurotoxin for the dissertation was that it would destroy central catecholaminergic fibers when administered cerebroventricularly, and the peripheral noradrenergic fibers would be depleted by way of bilateral ganglionectomies. It was not absolutely certain that intraventricular injections of 6-OHDA would destroy both central and peripheral noradrenergic fibers, especially since histology to check such destruction could not be done until at least one month after the injections were given. In addition, 6-OHDA was not given peripherally to destroy peripheral noradrenergic fibers. To guarantee that no noradrenergic innervation would be present, the MS + BG + 6-OHDA group was included.

Surgical Procedures

An injection of 6-OHDA was given to normal control animals to determine the effect it would have on recording spontaneous firing rates in the medial habenulae. At least

one month before recording or histological processing, an injection of 6-OHDA (300 ug) dissolved in 50 ul of saline with 1% ascorbic acid was given intraventricularly to the appropriate groups (18).

For placement of septal lesions and bilateral ganglionectomies, rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Bilateral septal lesions were made electrolytically with a Kopf stereotaxic instrument with the skull parallel to the horizontal plane. For the medial septal lesions, the electrode was lowered 6 mm ventral to the skull surface, 1 mm anterior to Bregma, and +/- 0.25 mm on either side of the midline. Bilateral lesions were made electrolytically by passing 2 mA for 20 seconds through a stainless steel electrode. The sham-operated animals underwent the same surgical procedure as did the septal lesioned animals, except that no current was passed through the electrode. Successful ganglionectomy was assessed by the presence of Horner's syndrome, consisting of ptosis (drooping of the upper eyelid) and enophthalmos (recession of the eyeball within its orbit) (2).

Electrophysiological Procedures

Electrophysiological recording was done 4 - 7 weeks post-surgery in a Kopf stereotaxic instrument under chloral hydrate anesthesia (300 mg/kg, i.p.). The habenulae (for recording) were approached stereotaxically using the

following co-ordinates: 3.5 mm posterior to Bregma, and 6 mm below the skull surface at the midline. Extracellular single unit responses were recorded with tungsten microelectrodes (4 - 10 M ohm), and stored on magnetic tape using conventional equipment (1). Only well-isolated, non-injured cell responses were included. Criteria used were stability of action potential amplitude and duration, and the ability to move the electrode without disturbing the extracellular response. Each cell was monitored for at least five minutes for stability, and analysis was based on examination of a 50 second segment taken from a 3 minute taped record. The recorded data were later played back into a microcomputer system (Polymorphic Systems, System 8813) which timed the interspike intervals (100 us resolution), and stored them in disk files for analysis of firing rates (1). A BASIC data analysis program that operates on the disc files generates latency (post-stimulus), interval and instantaneous frequency histograms on a video display with hard copy options (1). The latency histogram is a frequency histogram of the number of impulses as a function of time. The interval histogram is a frequency histogram of the distribution of interspike intervals by size. Instantaneous frequency is a bar graph plot of the reciprocal interspike interval as a function of sequential interval number (1). It is used primarily to characterize periodicity in neuronal firing, such as responses that are associated with non-bursting, regular

bursting and irregular bursting cell types (1).

Histological Procedures

At the conclusion of the experiments, animals were decapitated, and the brains quickly removed. Septal lesions and recording tracks were reconstructed from frozen sections (30 um) stained with cresyl violet.

Six animals, one from each group, were used for AChE histochemistry. Even though AChE is actually one of several cholinesterases, it is the one which splits acetylcholine most rapidly, and exerts its action at cholinergic synapses (48). The modified method of Naik (48) was used. The substrate was acetylthiocholine iodide, because this is hydrolyzed by AChE more rapidly than is acetylcholine. The acetyl substrate is hydrolyzed by both AChE and cholinesterase, so the positive reaction obtained by this substrate shows the total cholinesterase. To differentiate between the two enzymes, selective inhibitors are used. In this method, ethopropazine hydrochloride was used as an inhibitor of cholinesterase, because it does not affect the solutions and its inhibitory action is reliable.

A total of eight animals (at least one from each group) was prepared for determination of the presence or absence of sympathohabenular sprouting by catecholamine histofluorescence, and were sacrificed 4 - 7 weeks post-surgery. The glyoxylic acid method (SPG) of de la Torre

and Surgeon, and a Zeiss Fluorescence Photomicroscope
System were used to visualize catecholamine fibers
(15,16,17). To be more specific, unfixed frozen sections
(20 um) from the rat brain are mounted on microscope slides
and exposed for three seconds to a room temperature solution
containing sucrose-potassium phosphate-glyoxylic acid
(sucrose - 0.200M; potassium phosphate - 0.236M; glyoxylic
acid - 1% solution; pH = 7.4; SPG), which has a high
specific sensitivity for monoamines. The tissues are air
dried and then heated in an oven for 5 minutes. The
average processing time from fresh tissue to microscopic
examination takes about 20 minutes.

Data Analysis

Pair-wise statistical comparisons between the six
experimental group utilized the Kolmogorov-Smirnov test
(58). The following formula,

$$X^2 = 4D^2 \frac{n(1)\, n(2)}{n(1) + n(2)}$$ has a sampling distribution which

is approximated by a chi-square distribution with two
degrees of freedom. The abbreviations $n(1)$ and $n(2)$ refer
to the first and second sample, respectively. The summary
of the procedure is as follows. First, the two groups of
scores being considered are arranged in a cumulative
frequency distribution, using the same intervals or
classifications for both distributions. Second, the
difference between the two sample cumulative distributions

at each listed point is determined. Third, the largest of
the differences is determined, which is D. Finally, for a
one-tailed test where n(1) and n(2) are considered, the
value of X^2 with df = 2, associated with the observed D is
computed from the previous formula. The significance of
the resulting value is determined by reference to a table
of critical values of Chi Square. The Kolmogorov-Smirnov
test is sensitive to any kind of difference in the
distributions from which the two samples were drawn, such
as central tendency, dispersion and skewness.

CHAPTER III

RESULTS

All animals were allowed to recover for at least four weeks following surgery. This period allows time for sufficient sympathohabenular enrichment in the septal lesioned group (4). The electrophysiological recording procedure examines baseline firing rates, and the pattern of firing in medial habenular neurons under sympathetic enriched and depleted conditions.

Types of Responses

Among the 279 spontaneously active cells recorded in all groups, there were two distinct categories depicting two types of cellular responses: non-bursting (69%), and bursting (31%). These response types were observed in all groups, regardless of treatment, but not in the same proportion. The bursting cell category was further sub-divided into regular and irregular bursting responses. Figure 1 shows examples of non-bursting, regular bursting and irregular bursting cells as they appeared on the oscilloscope when recording was being conducted. The non-bursting cell (Figure 1A) is characterized by firing single spikes continuously at varying rates. A regular bursting cell is shown in Figure 1B. One characteristic of this type of cell is that the intervals between the spikes in the burst are relatively constant. Figure 1C is an example

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Fig. 1. Examples of extracellular single unit response types recorded in the medial habenular region. A. a continuously firing, non-bursting cell. B. a regular bursting cell. C. an irregular bursting cell.
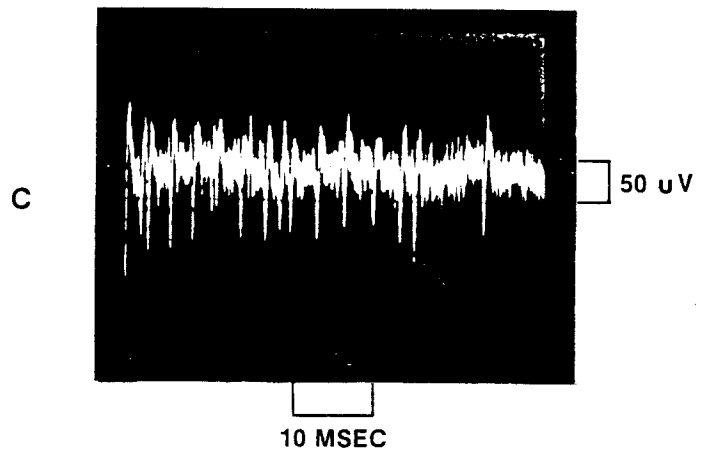
NON-BURSTING CELL

A

50 uV

10 MSEC

REGULAR BURSTING CELL

B

50 u V

10 MSEC

IRREGULAR BURSTING CELL

C

50 u V

10 MSEC
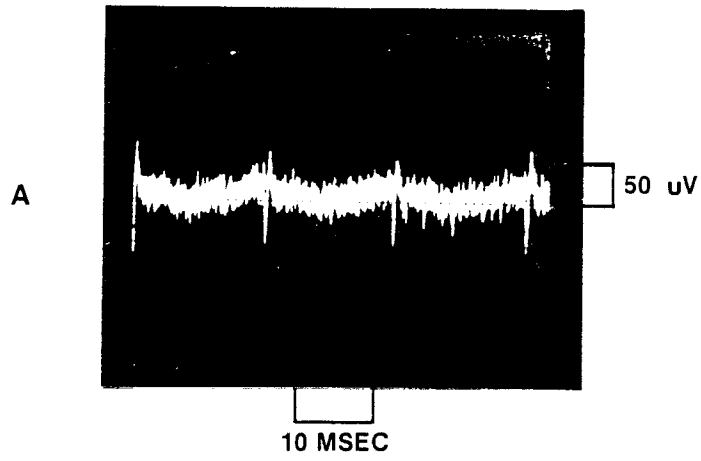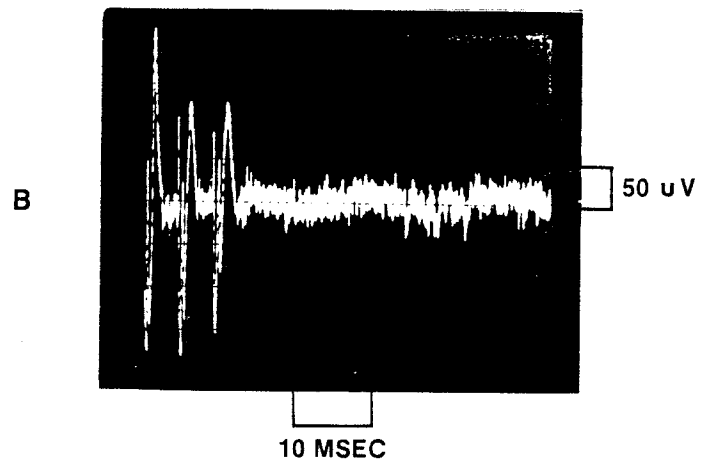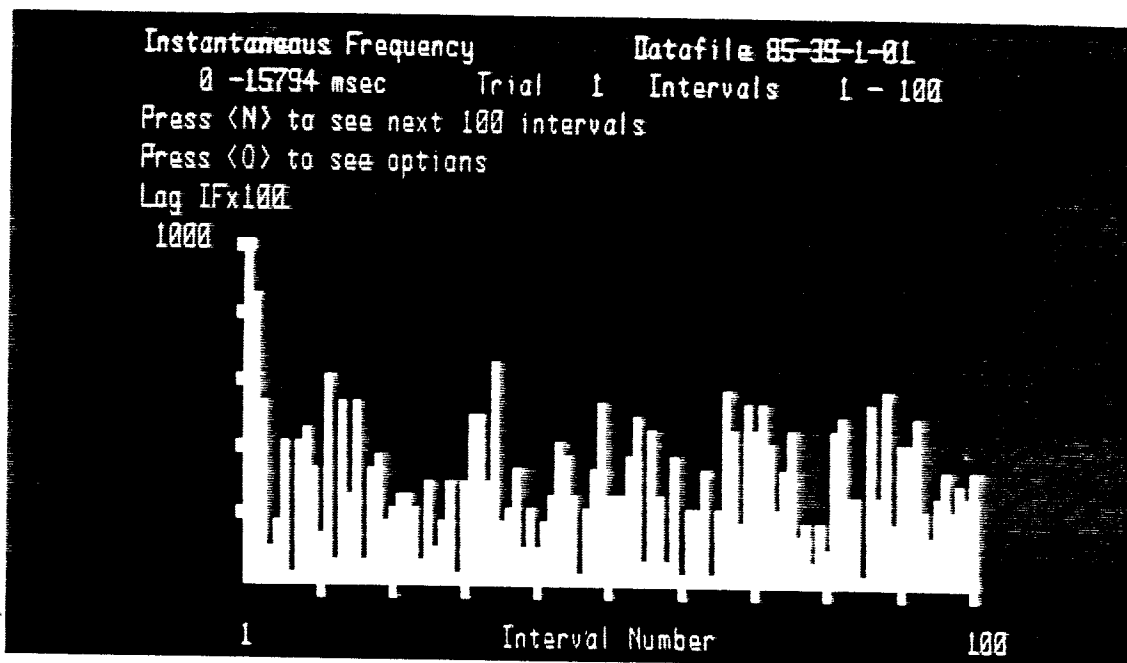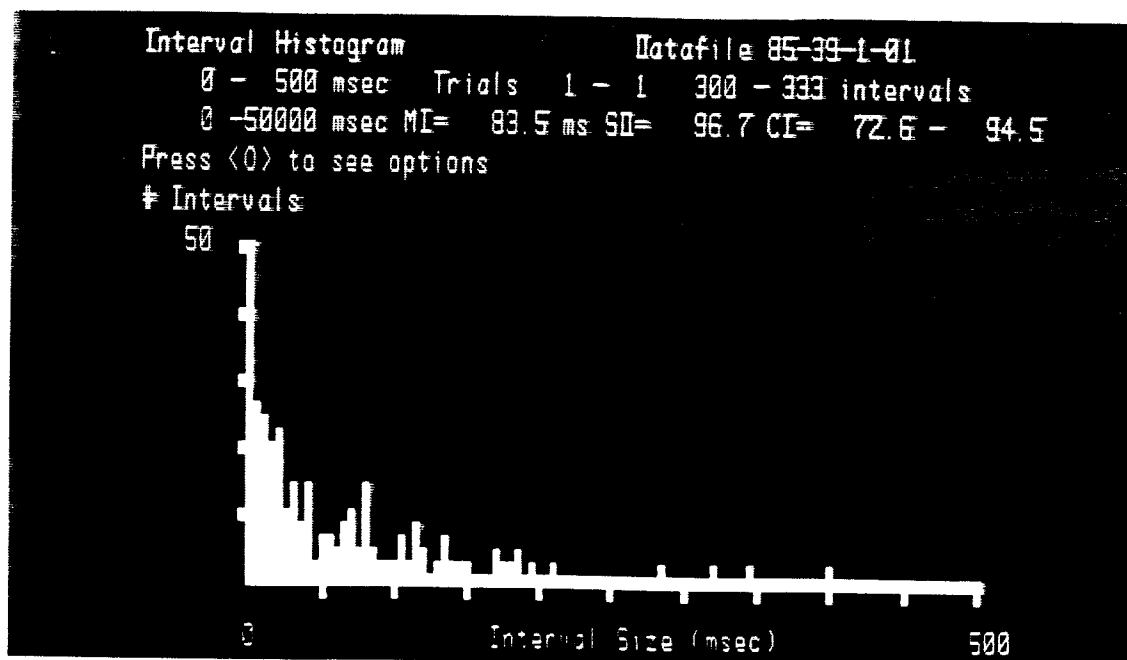
of an irregular bursting cell, a characteristic of which is
that both the number of spikes in the burst and the
intervals between the spikes in the burst are variable.

The firing characteristics of non-bursting, regular
bursting and irregular bursting cells can be seen from the
Instantaneous Frequency (IF) and the Interspike Interval
Histograms (IH). Examples of the IF and IH histograms are
shown in Figures 2, 3 and 4. The IF for a non-bursting cell
(Figure 2A) shows a periodic variation in instantaneous
frequency. The skewed shape of the IH for this cell is
typical of cells in the non-bursting (Figure 2B)
category. The IF plot for the regular bursting cell
(Figure 3A) shows the characteristics for this group, in
that there is little variation in the height of each bar,
and the spacing between the bars in the histogram. This
plot shows a predominance of frequencies at a constant
value, indicating that when the cell fires a burst of spikes,
the intervals within the burst are relatively constant, and
the intervals between the burst are also relatively constant.
The IH of the same regular bursting cell is shown in Figure
3B. This histogram shows most of the intervals tightly
clustered around the mean value, which in this case is 78.3
msec. Interburst intervals longer than 500 msec. do not
appear in this interval histogram. The IF of a typical
irregular bursting cell (Figure 4A) shows a much wider
variation of intra- and inter-burst intervals, as observed
in both the amplitude and spacing between the bars in the

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Fig. 2. The firing characteristics of non-bursting and
bursting cells, as shown in their profile on Instantaneous
Frequency (IF) (2A) plots and Interval Histograms (IH)
(2B). This figure depicts a non-bursting cell.

Instantaneous Frequency   Datafile 85-39-1-01
     0 -15794 msec   Trial 1   Intervals   1 - 100
Press <N> to see next 100 intervals
Press <O> to see options
Log IFx100
1000

1   Interval Number   100

A

Interval Histogram   Datafile 85-39-1-01
     0 - 500 msec   Trials 1 - 1   300 - 333 intervals
     0 -50000 msec MI=   83.5 ms SD=   96.7 CI=   72.6 -   94.5
Press <O> to see options
Intervals
50

0   Interval Size (msec)   500

B

43

Fig. 3. The instantaneous frequency histogram (3A) and the interval histogram (3B) for a regular bursting cell.
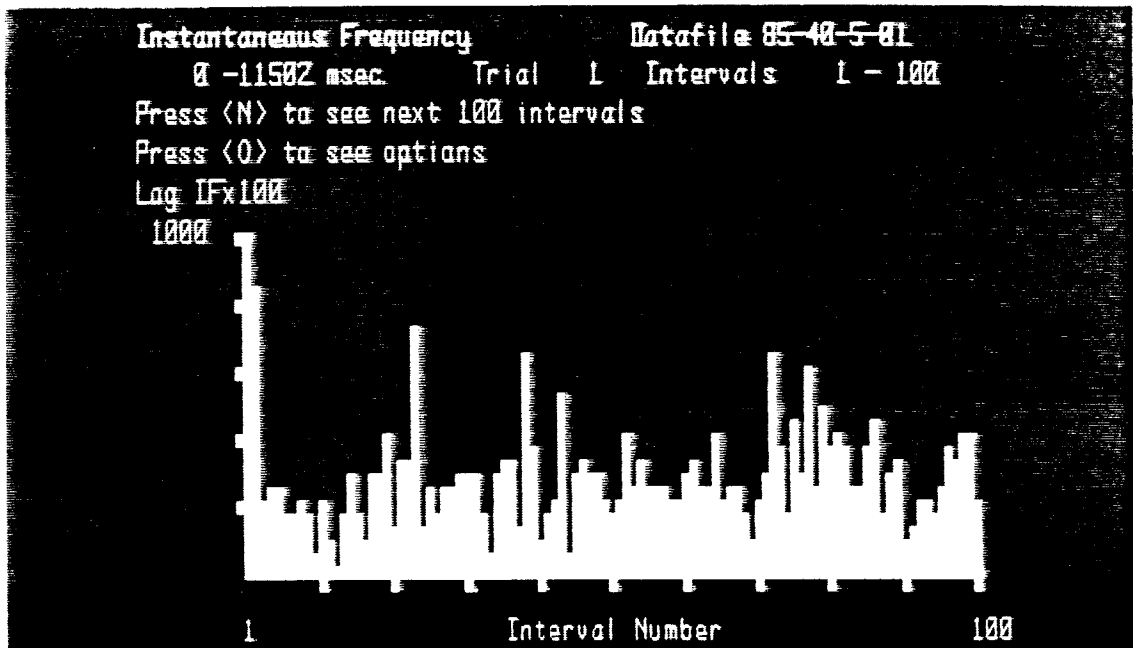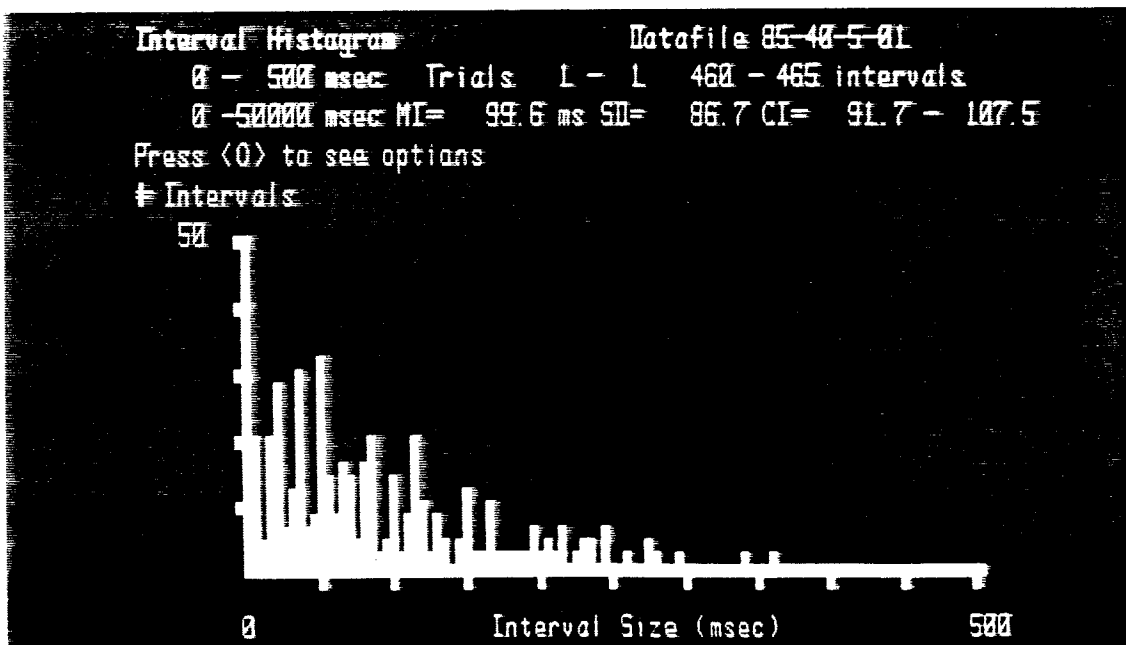
A

B

45
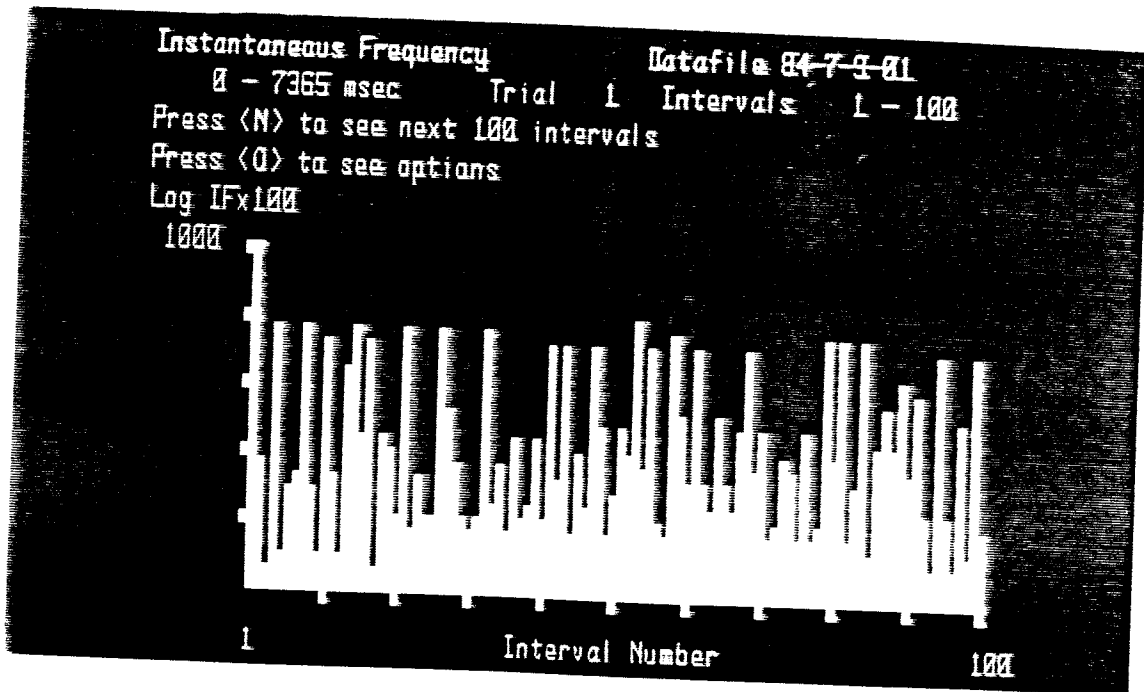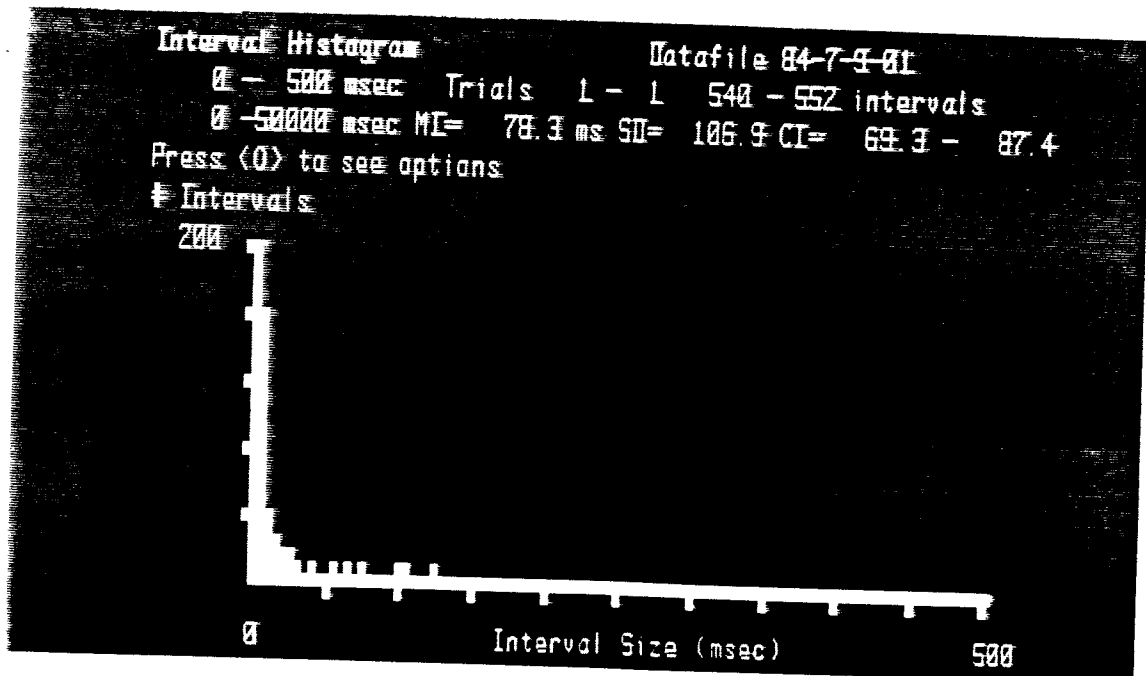
Fig. 4. The instantaneous frequency (4A) plot and the interval histogram (4B) for an irregular bursting cell.

A

B

histogram. The IH for the same irregular bursting cell
is displayed in Figure 4B. Again, most of the intervals are
clustered around the mean value, which is 99.6 msec.

The distribution of these cell types among all groups
shows an interesting pattern, which is summarized in Table
I. The control group consisted of the two response types
being in almost equal proportion, with the non-bursting
cells accounting for 54% of the total, and the bursting
cells accounting for 46%. The medial septal lesioned
group showed a slightly more equal balance; 51% of the
cells were of the non-bursting type, and 49% were
characterized as bursting. These two groups showed the
most equal proportion of non-bursting and bursting cell
types. They are also the only two groups with intact
central and/or peripheral noradrenergic innervation. When
the next four groups in the table are examined, it will be
noticed that in each group, either central, peripheral or
both kinds of noradrenergic input are removed. It is
apparent that a significant increase in non-bursting cells
occurs in those groups that received a 6-OHDA lesion or a
bilateral ganglionectomy or both. This significant
difference was examined statistically by the Chi-Square
One-Sample Test (58). This statistical test ascertained
that the four groups of animals with either central or
peripheral Ne depletion had significantly higher ratios of
non-bursting to bursting cells when compared with either
the control or medial septal lesioned group. Therefore, it

TABLE I

PERCENTAGE OF ALL CELL RESPONSE TYPES

	Non-Bursting		Bursting	
Control	54 %		46 %	
		Irregular		Regular
		83 %		17 %
Medial Septal	51 %		49 %	
		Irregular		Regular
		82 %		18 %
MS + BG	69 %		31 %	
		Irregular		Regular
		56 %		44 %
Sham + 6-OHDA	83 %		17 %	
		Irregular		Regular
		100 %		0 %
MS + 6-OHDA	85 %		15 %	
		Irregular		Regular
		100 %		0 %
MS + BG + 6-OHDA	95 %		5 %	
		Irregular		Regular
		33 %		67 %

is suggested that the change in the non-bursting to bursting cell ratio is related to depletion of norepinephrine.
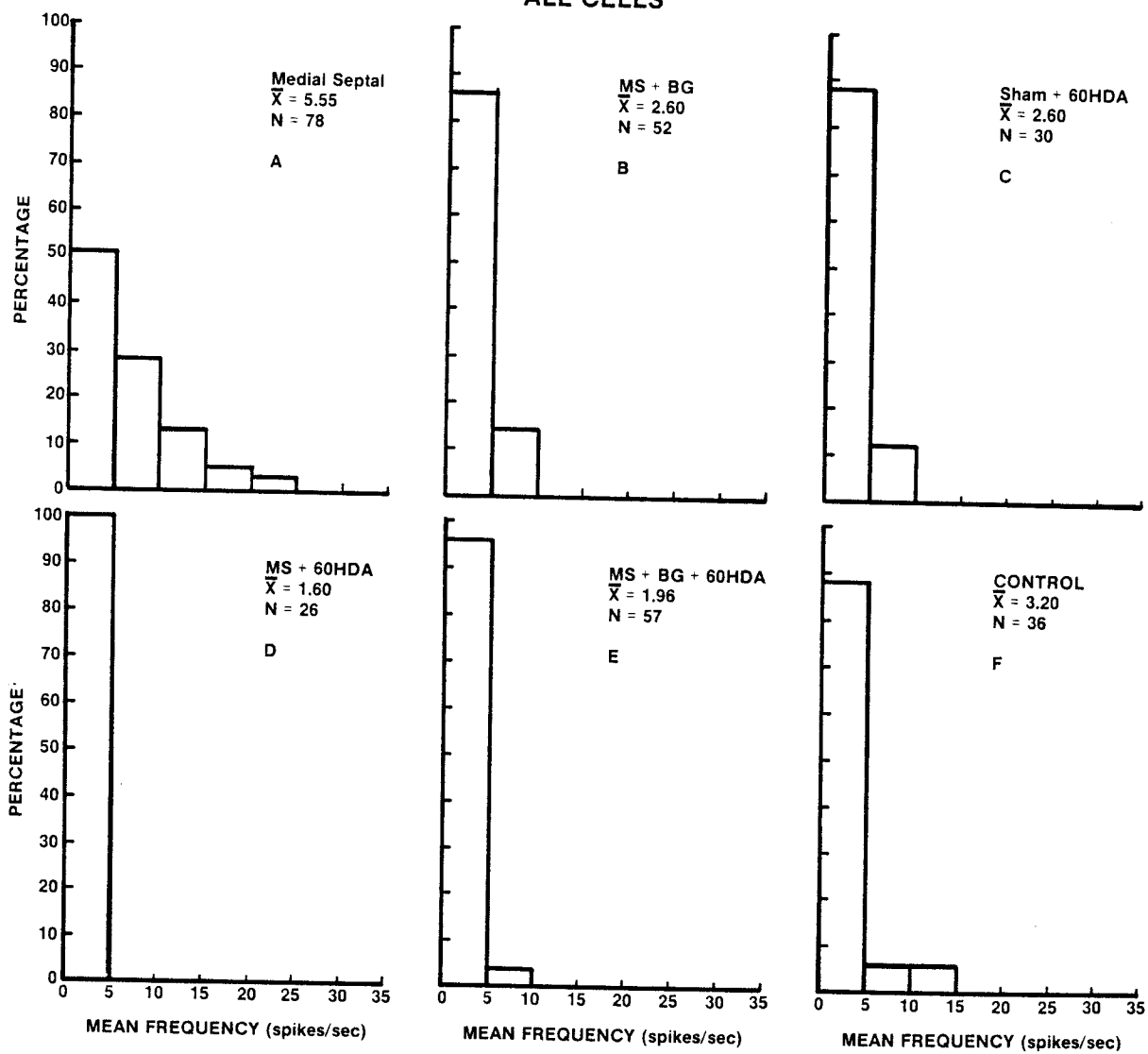
Bursting cells were further sub-divided into irregular (78%) and regular (22%) types for all six groups, although each separate group has a different proportion of these sub-types. Again referring to Table I, the bursting cells of the control group have 83% irregular types, and 17% regular types of cells. In the medial septal group, 82% of the bursting cells were irregular, and 18% were regular. A remarkable similarity between cell type ratios is seen with these two groups, even though the presently discussed ratio deals with irregular to regular bursting cell types. The only clear-cut distinction to be made among the next four groups is that both the Sham + 6-OHDA and the MS + 6-OHDA groups had only irregular bursting cells. It should be pointed out that the non-bursting to bursting cell ratio between these two groups is also very similar.

Spontaneous Activity

A comparison of spontaneous firing rates for all the cells in all six groups is shown in Figure 5. The spontaneous firing rates in the medial septal group (5A) were found to be significantly higher than every other group. When compared with the MS + BG group (5B) (no peripheral ingrowth), the mean firing rates were significantly higher (p < .01) in the septal lesioned group

Fig. 5. Distribution of spontaneous firing rates for all
cells in all groups. Bar height on the ordinate represents
the percentage of cells in each bin.

DISTRIBUTION OF FIRING RATES
ALL CELLS

A Medial Septal
$\overline{X}$ = 5.55
N = 78

B MS + BG
$\overline{X}$ = 2.60
N = 52

C Sham + 60HDA
$\overline{X}$ = 2.60
N = 30

D MS + 60HDA
$\overline{X}$ = 1.60
N = 26

E MS + BG + 60HDA
$\overline{X}$ = 1.96
N = 57

F CONTROL
$\overline{X}$ = 3.20
N = 36

PERCENTAGE

MEAN FREQUENCY (spikes/sec)

(with sympathohabenular ingrowth). The septal lesioned
group displayed spontaneous firing rates 30% higher than
those displayed in the MS + BG group. The septal lesioned
group of animals also showed significantly higher (30%)
rates of firing when compared with rates in the Sham + 6-OHDA
(no central innervation, p < .01), (5C). Likewise, when
compared with the MS + 6-OHDA group (no peripheral or
central innervation), the spontaneous firing rates of
septal animals were 39.5% higher (p < .001), (5D). In
addition, the firing rates were significantly higher
(35.9%) when compared withh the MS + BG + 6-OHDA group (no
peripheral or central innervation, p < .001), (5E).
Finally, the firing rates of septal lesioned animals were
23.5% higher than the firing rates of normal control
animals (p < .01, 5F). The firing rates of the septal
lesioned group are significantly higher than all other
groups, but none of the other groups are significantly
different from each other or the control group.

The effect of peripheral and/or central sympatho-
habenular ingrowth, or no ingrowth was also examined in non-
bursting and bursting cell types. There are large
differences in the proportion of non-bursting versus
bursting cell types in the different groups, and it was
felt the differences should be examined more closely.
Since the firing rates of septal lesioned animals were
significantly higher than those in all other groups, this
higher percentage could be represented by a higher

percentage of either non-bursting or bursting cell types. The histograms for the rates of non-bursting cells in the six groups are shown in Figure 6.

Rates of non-bursting cells in animals with medial septal lesions (Figure 6A) are significantly higher than the rates of non-bursting cells in all other groups, except for one, the Sham + 6-OHDA group (Figure 6C). When the septal lesioned group and the Sham + 6-OHDA group were compared statistically by the Kolmogorov-Smirnov test, the p value was < .10, but the variant shapes of the histograms indicate that if the number of cells in each group had been larger, the p value would have been significant. The firing rates of non-bursting cells from all the other groups were not significantly different from one another, or from the control group.

Figure 7 examines the bursting cell responses among all six groups. The rates of these cells in animals with medial septal lesions are not different from the rates of bursting cells in the MS + BG group, the normal control group, the Sham + 6-OHDA group, the MS + 6-OHDA group, or the MS + BG + 6-OHDA group. Even though examination of the histograms in Figure 7 would not suggest that the firing rates of the septal lesioned group were no different from those of the other groups, again, the very small number of bursting cells in each of the other groups prohibited the differences from being statistically significant.

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Fig. 6. Distribution of spontaneous firing rates for non-bursting cells in all groups.

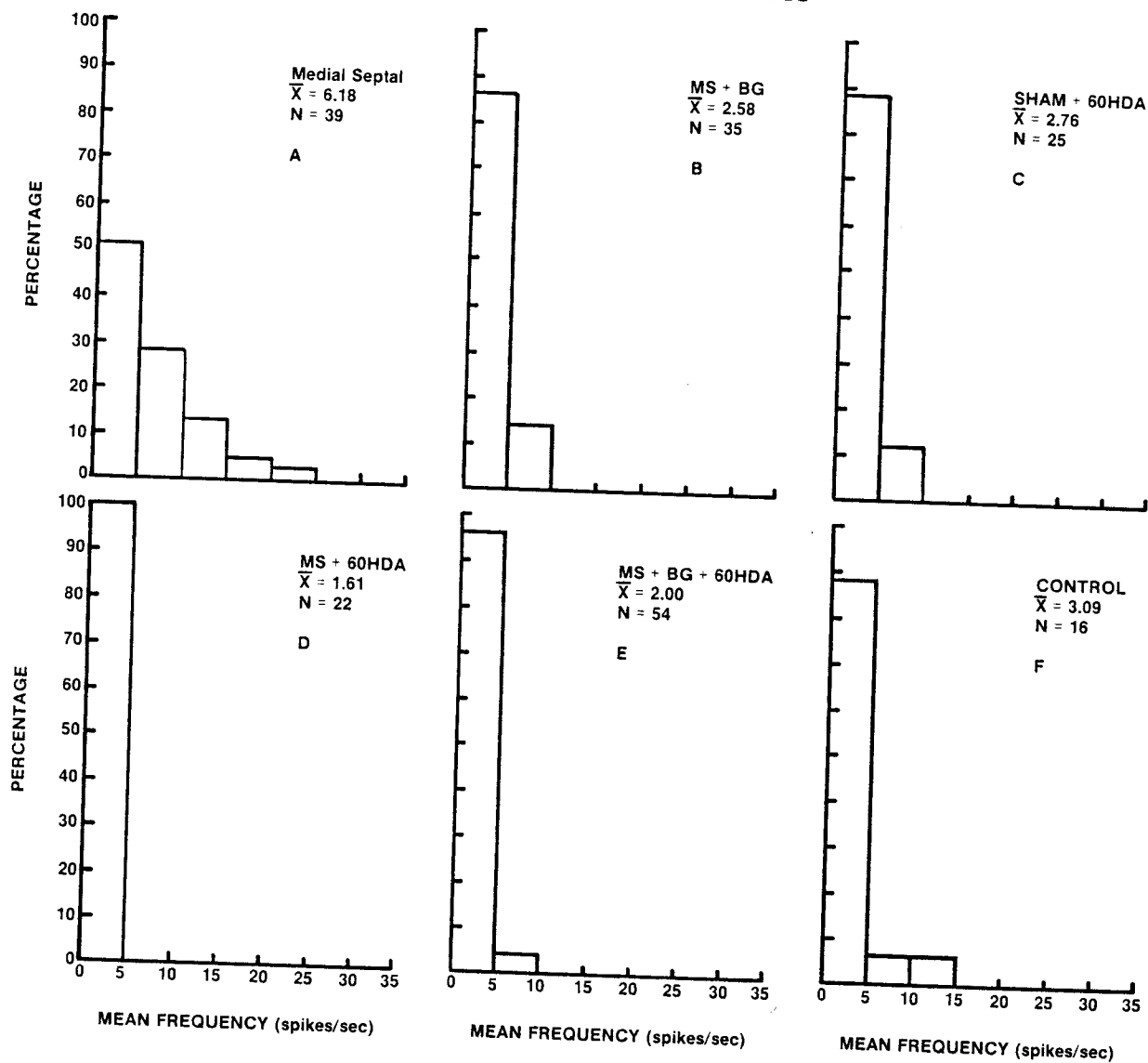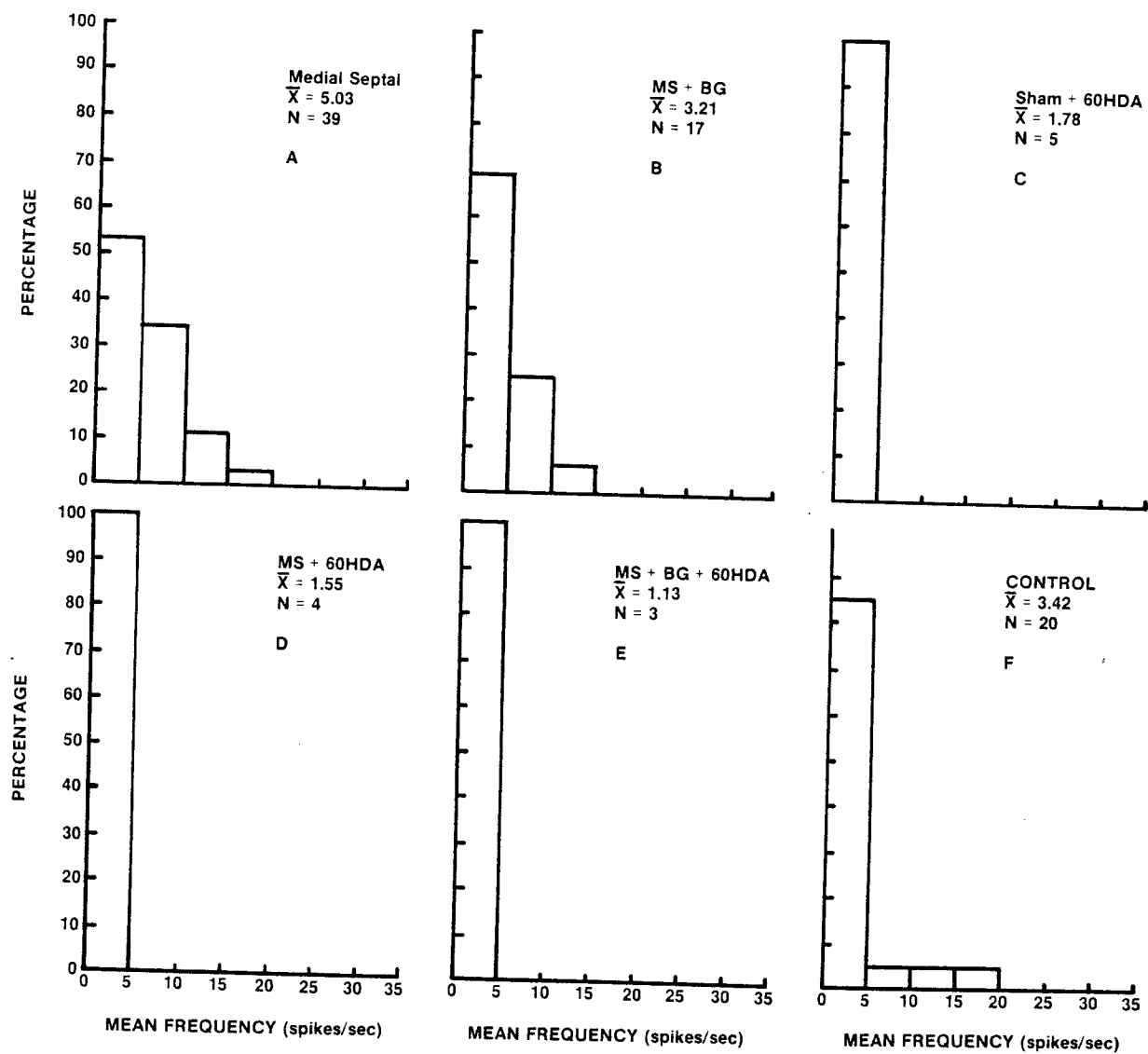DISTRIBUTION OF FIRING RATES
NON-BURSTING CELLS

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Fig. 7. Distribution of spontaneous firing rates for
bursting cells in all groups.

DISTRIBUTION OF FIRING RATES
BURSTING CELLS

PERCENTAGE

Medial Septal
$\overline{X}$ = 5.03
N = 39

A

MS + BG
$\overline{X}$ = 3.21
N = 17

B

Sham + 60HDA
$\overline{X}$ = 1.78
N = 5

C

PERCENTAGE

MS + 60HDA
$\overline{X}$ = 1.55
N = 4

D

MS + BG + 60HDA
$\overline{X}$ = 1.13
N = 3

E

CONTROL
$\overline{X}$ = 3.42
N = 20

F

MEAN FREQUENCY (spikes/sec)

MEAN FREQUENCY (spikes/sec)

MEAN FREQUENCY (spikes/sec)

The rates of bursting cells in the septal lesioned group are significantly higher than the rates of non-bursting cells in all the other groups, except the control group and the septal lesioned group.

Table II is a synopsis of non-bursting versus bursting data after statistical comparisons were made. Two points to emphasize when examining this table are (1) the bursting cell proportion decreases as the central and peripheral noradrenergic innervation is removed, and (2) because the medial septal lesioned group was the only group with significantly different firing rates from the others, only the firing rates from this group were sub-divided into non-bursting and bursting types for statistical comparisons using the Kolmogorov-Smirnov test.

The increase in spontaneous activity among animals with sympathohabenular ingrowth (septal lesions) occurred in both non-bursting and bursting cell types (Table II) when compared with non-bursting cells of the control, MS + BG, MS + 6-OHDA, and MS + BG + 6-OHDA groups. When compared with the Sham + 6-OHDA group, only the increase in spontaneous activity of the bursting cells (MS group) accounted for the difference between the two groups. Finally, when the MS group was compared with both non-bursting and bursting cell types of the control group, only the non-bursting cells of the septal lesioned group accounted for the increase in spontaneous activity.

TABLE II

COMPARISON OF SEPTAL LESIONED GROUP WITH ALL OTHER GROUPS:
BURSTING AND NON-BURSTING CELLS

CHI-SQUARE VALUES FROM KOLMOGOROV-SMIRNOV TEST

Medial Septal Lesioned Group

	Non-Bursting Cells	Bursting Cells
Control		
Non-Bursting	6.24 (p<.05)	5.52 (NS)
Bursting	6.08 (p<.05)	5.37 (NS)
MS + BG		
Non-Bursting	9.04 (p<.02)	8.02 (p<.02)
Bursting	1.48 (NS)	1.13 (NS)
Sham + 6-OHDA		
Non-Bursting	5.18 (NS)	7.39 (p<.05)
Bursting	4.26 (NS)	3.89 (NS)
MS + 6-OHDA		
Non-Bursting	13.50 (p<.01)	12.26 (p<.01)
Bursting	3.48 (NS)	3.19 (NS)
MS + BG + 6-OHDA		
Non-Bursting	18.34 (p<.001)	16.51 (p<.001)
Bursting	2.67 (NS)	2.48 (NS)

The spontaneous firing rates of each of the six groups reflect information received from latency histograms, which are frequency histograms of the number of impulses fired as a function of time. The latency histograms indicated how fast the cells fired in a particular time frame. The mean interval data comes from interval histograms, which are frequency histograms of the distribution of interspike intervals by size, without regard to their time of occurrence. The interval histograms indicate how long the intervals are between spikes. The faster a cell fires per unit of time, the more spikes there will be in that unit of time, and the shorter the intervals between the spikes will be in that same unit of time.

The mean interval data for the MS group shows this group has mean intervals 36% lower than the mean intervals of the Sham + 6-OHDA group, ($p < .05$). When the MS group was compared with the MS + 6-OHDA group, the septal lesioned group has mean intervals 50% lower ($p < .001$), and when compared with the MS + BG + 6-OHDA group, the mean intervals were 57% lower ($p < .001$). There were no significant differences when compared with the other groups.

In summary, there are two major points of consideration. First, the proportion of non-bursting and bursting cell types varied considerably among the experimental groups in this study. The important finding is that as central and peripheral noradrenergic input decreases, the ratio of non-bursting to bursting cells

increases dramatically. The second point is that the mean firing rates of the septal lesioned group are significantly higher than the firing rates of all other groups, which are not significantly different from one another. Both non-bursting and bursting cells accounted for the significantly higher firing rates of the septal lesioned animals when they were compared with the other groups.

Histological Results

A reconstruction of a representative septal lesion is shown in Figure 8. All the lesions were confined to the medial nuclei, in that areas posterior or ventral to the nucleus were not destroyed, and the lateral septal nuclei were not destroyed. In a few of the animals, the lesions started a little anterior to the medial septal nuclei, in that they started at the anterior level of the anterior commissure before the medial nuclei came into view.

A reconstruction of a representative electrode track for recording in the medial habenulae is shown in Figure 9A. All recording was done in the medial habenular region, as the histological results show that the electrode tracks were confined to that area. The electrode track for the injection of 6-OHDA into one of the lateral ventricles is shown by a representative example in Figure 9B. All of the 6-OHDA injections were correctly placed in one of the lateral ventricles; there were no intracerebral injections

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Fig. 8. Reconstruction of a representative example of a medial septal lesion. Drawings are based on the atlas of Konig and Klippel (35).
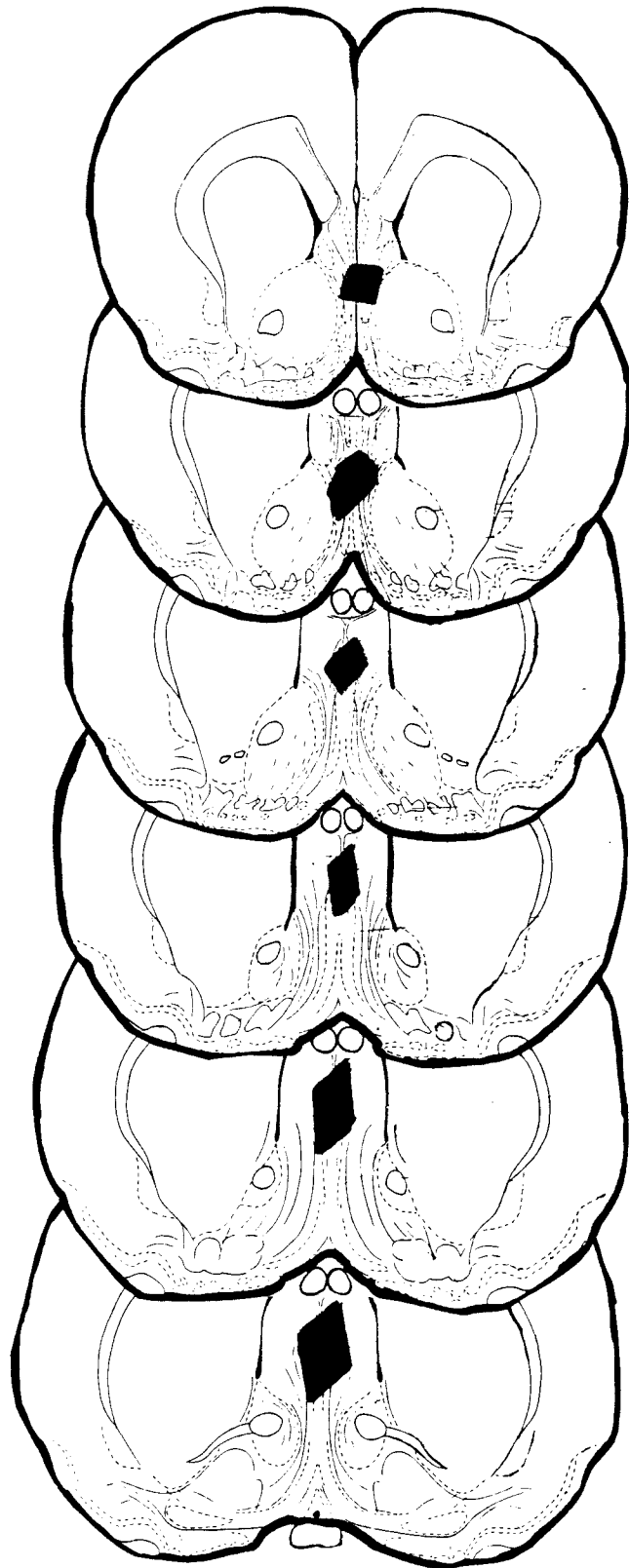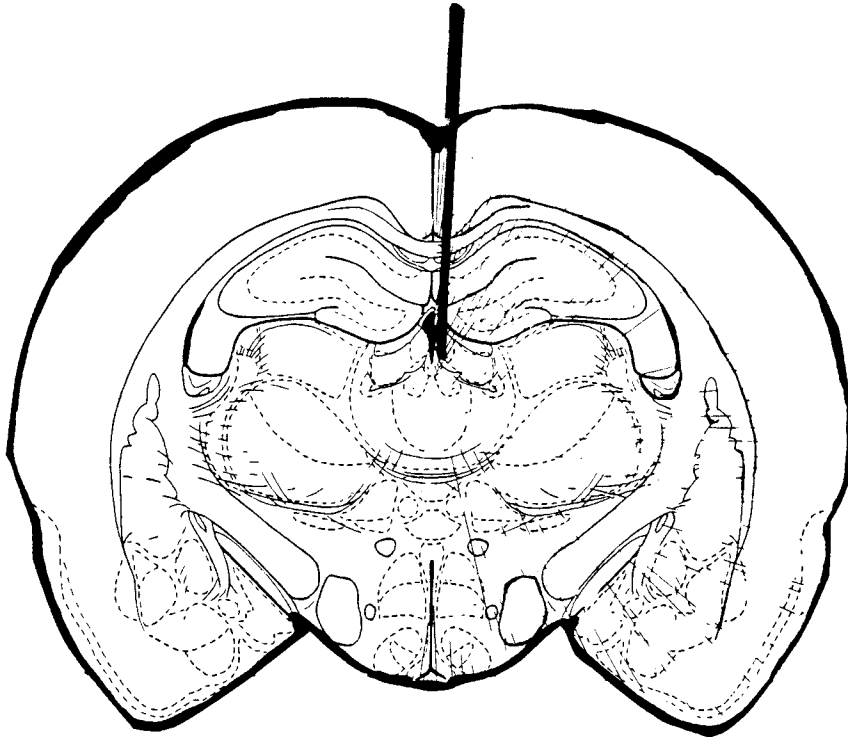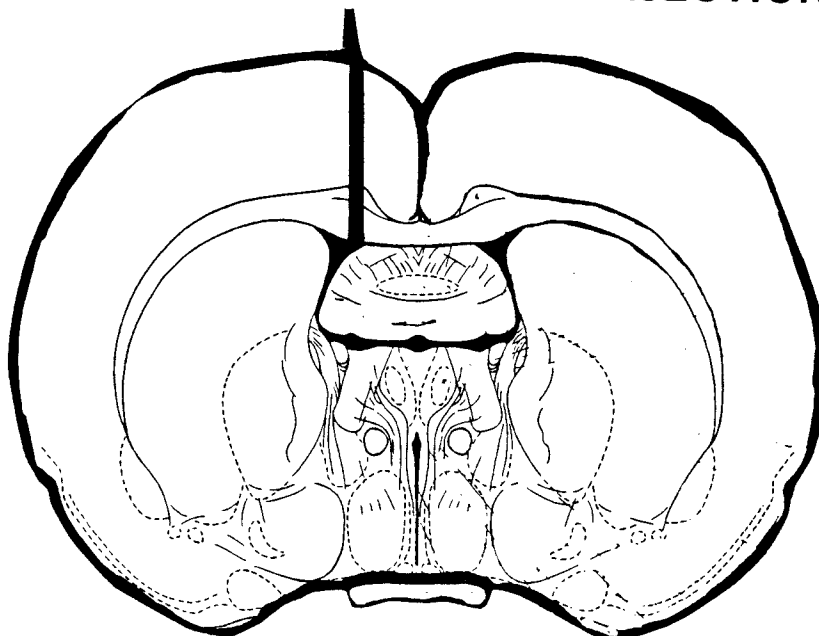
MEDIAL SEPTAL LESION

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Fig. 9. Reconstruction of representative examples of an electrode track in the medial habenular region (A.), and the electrode track for the injection of 6-OHDA in the lateral ventricle (B.). Drawings are based on the atlas of Konig and Klippel (35).

A. PLACEMENT OF RECORDING ELECTRODE


B. PLACEMENT OF 6-OHDA INJECTION

made accidentally.

The AChE histology corresponded well with expectations. In the normal control animal, no loss of acetylcholine was seen. The medial septal animals showed a profound loss of AChE staining in the hippocampus, and a moderate loss in the habenulae. Examples of the AChE histology are seen in Figure 10. Sections from the MS + BG, MS + 6-OHDA, and the MS + BG + 6-OHDA groups showed the same pattern of staining as the MS group, as would be expected. The Sham + 6-OHDA group showed the same staining as the normal control group. No loss was expected, as cholinergic denervation did not occur.

Considering the fluorescent histochemistry, a description of the fluorescence in the normal control animal will be discussed first. In this particular study, fibers characteristic of central and peripheral noradrenergic innervation were not seen in the medial habenular nuclei, which corresponds with one study (11), but not with others (3,4,65). In addition, the choroid plexus did not fluoresce brightly, but only in a very dim fashion. The only structure in the control animal with a high amount of fluorescence was the median eminence. This hypothalamic structure was used to judge the effectiveness of the 6-OHDA injection. As the median eminence is densely innervated by noradrenergic terminals, it would be expected to show a loss of catecholaminergic fluorescence if the 6-OHDA injection was effective. In the

Fig. 10. Examples of AChE histology in a control animal
(10A), and in a medial septal animal (10B), with cholinergic
denervation. The coronal sections shown in this figure
were cut at the level of the habenular nuclei,
approximately 3.5 mm posterior to Bregma. Abbreviations:
mh, medial habenulae; lh, lateral habenulae; h,
hippocampus; 3V, third ventricle; cp, choroid plexus;
t, thalamus.
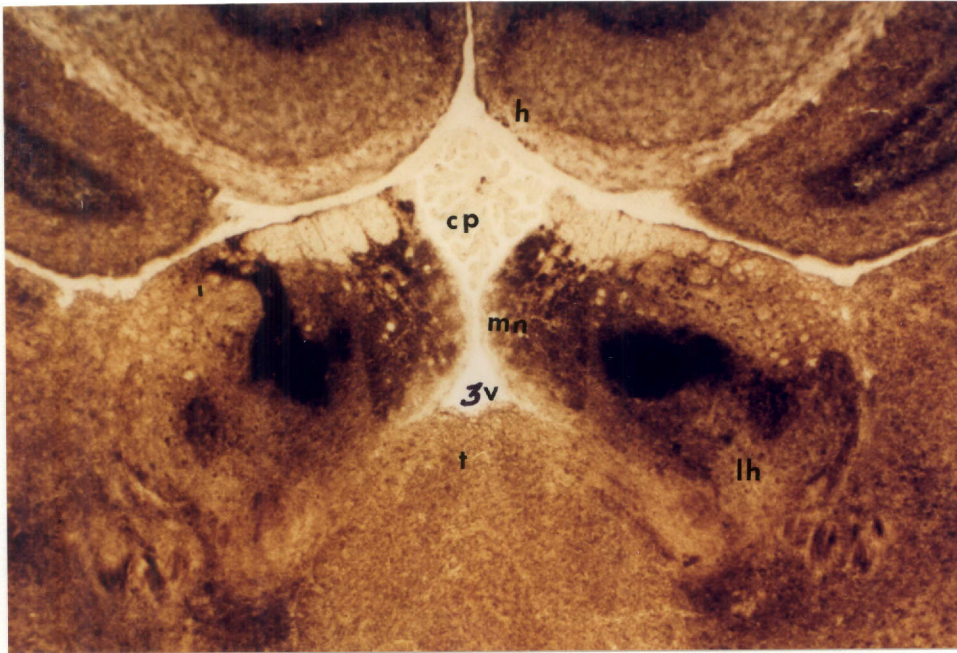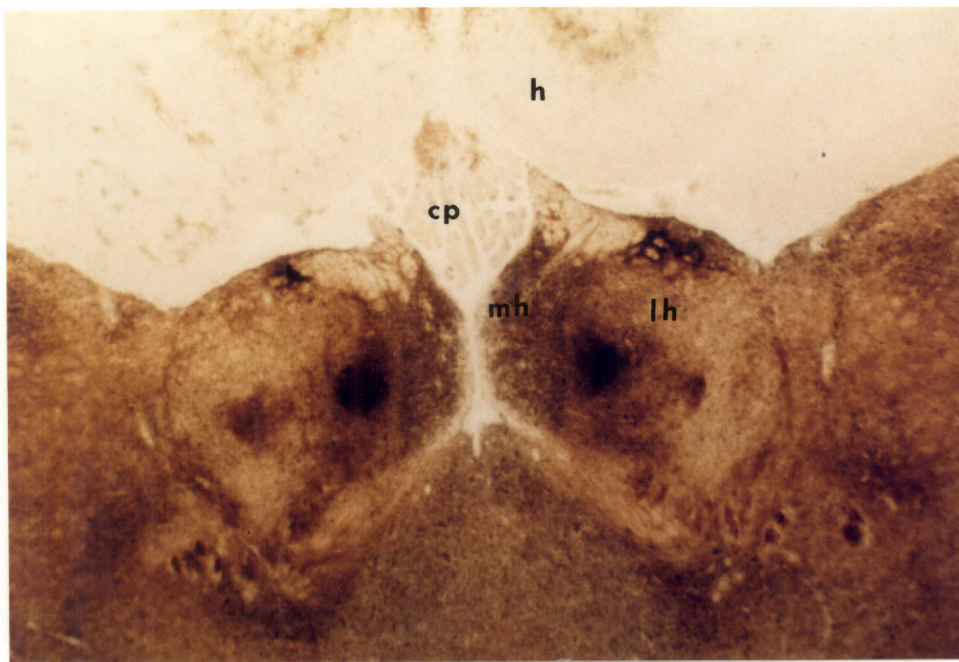
medial septal lesioned animal, fluorescence was seen in the
medial habenular nucleus, and the amount of fluorescence
increased in the choroid plexus, as would be expected
because of sympathohabenular ingrowth. There was no
fluorescence seen in the medial habenulae or choroid plexus
in the MS + BG group. Examples of the fluorescence seen in
the MS and the MS + BG groups are shown in Figure 11. In
the MS + 6-OHDA group, the median eminence, choroid plexus,
and medial habenulae did not fluoresce. The Sham + 6-OHDA
group also showed a decreased amount of fluorescence in
the median eminence. The last group, MS + BG + 6-OHDA,
showed no fluorescence in the medial habenulae, choroid
plexus or median eminence. Only the septal lesioned
animals showed fluorescent evidence of peripheral
sympathetic ingrowth. Unfortunately, no central
innervation of the medial habenulae was recognized either
before or after septal lesions. It cannot be stated
histochemically that central noradrenergic fibers increased
their innervation of the medial habenular nuclei in
response to cholinergic denervation.

To review the histological results, the normal animal
showed strong AChE staining, but no fluorescent staining in
the medial habenulae. This indicates the cholinergic
system is intact in a normal animal, but no sympathetic
noradrenergic fibers could be seen. A septal lesioned
animal showed loss of AChE staining in the medial
habenulae, and an increase in catecholamine fluorescence in

that area. These histological results reflect cholinergic denervation and sympathohabenular innervation. All other groups were given either a 6-OHDA lesion, a bilateral ganglionectomy, or both, and no fluorescent staining or AChE loss was seen in those animals.

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Fig. 11. Examples of catecholamine fluorescence in animals
with and without sympathohabenular ingrowth. A.
Appearance of the right habenular nucleus and choroid
plexus after a medial septal lesion (sympathohabenular
ingrowth). B. Appearance of the right habenular nucleus
after a medial septal lesion and a bilateral ganglionectomy
(no sympathohabenular ingrowth). The coronal sections
shown in this figure were cut at the level of the habenular
nuclei, approximately 3.5 mm posterior to Bregma.
Abbreviations: h, habenulae; 3V, third ventricle; cp,
choroid plexus.

A

cp

h

B

h
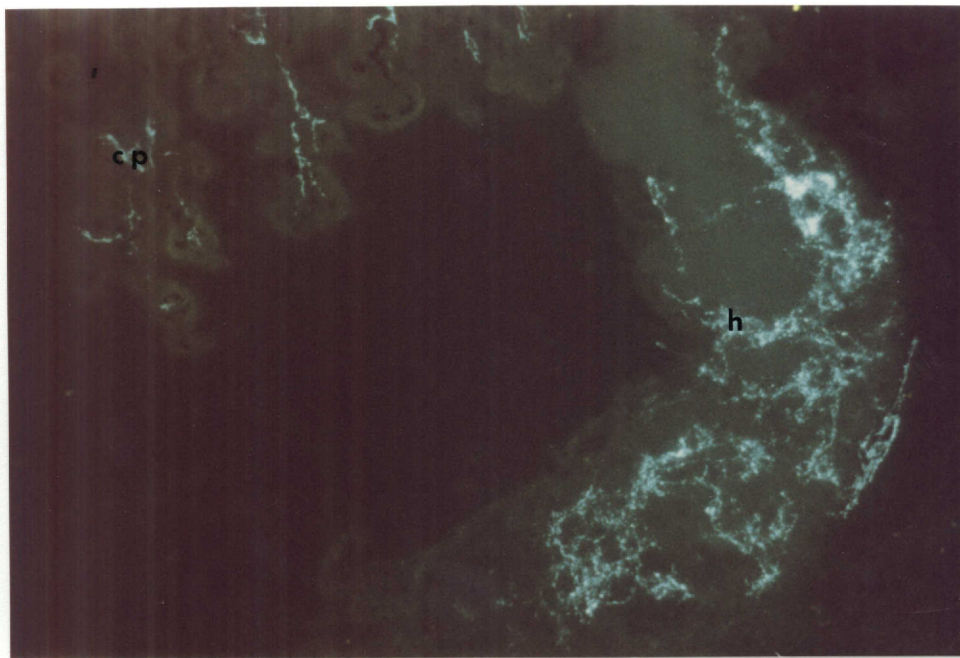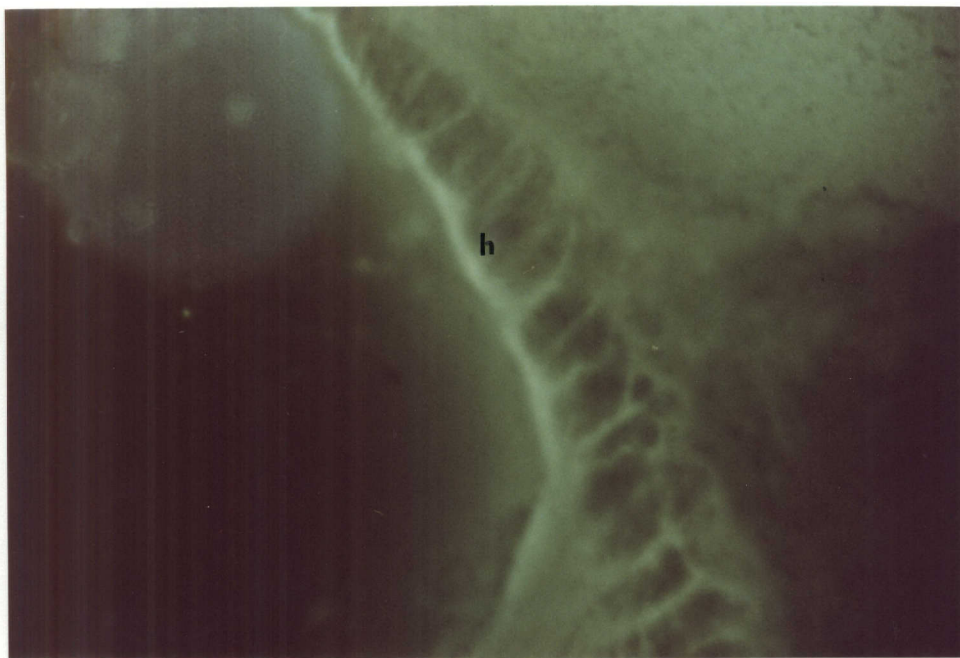
CHAPTER IV

DISCUSSION

The results suggest that the growth of sympathetic terminals into the medial habenulae following medial septal lesions has some functional significance with respect to spontaneous activity of the firing cells. Mean firing rates in animals with medial septal lesions (sympatho-habenular ingrowth) are significantly different from control animals, animals with no sympathohabenular ingrowth (MS + BG, MS + 6-OHDA and MS + BG + 6-OHDA), and control animals with central noradrenergic innervation removed (Sham + 6-OHDA). No significant differences were found when the mean firing rates of the other groups were statistically compared. This strongly suggests that the existence of the sympathohabenular ingrowth in the septal lesioned animals is responsible for the significant difference in firing rates between this group and the other groups. In each comparison, the firing rates of the septal lesioned animals are significantly higher than the firing rates of animals in the other groups, meaning the medial habenular cells fired faster in the septal lesioned group.

It was established that the cellular recording in the medial habenular nuclei was distributed between two types of cells, non-bursting and bursting, which were

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representative of spontaneous activity. Animals with

septal lesions have an almost equal distribution of cell

types in the medial habenular nuclei. The only other

group with a distribution of cell response types that

approximated the one held by the septal lesioned group was

the control group. All other groups had a disproportionate

distribution, with the cell response type weighing heavily

in favor of non-bursting cells. It is apparent that the

ratio of non-bursting to bursting cells increases

considerably as central and/or peripheral noradrenergic

innervation is removed. This would indicate that the

increase in non-bursting cells in these groups is related

to the depletion of norepinephrine. The mechanism for the

increase in non-bursting cells (or the decrease in bursting

cells) in the medial habenulae is not understood, but

warrants further study.

Grace and Bunney (27) have examined single spike

firing in nigral dopamine (DA) neurons. Spontaneously

discharging DA cells fire in two different patterns, a

slow, single spiking pattern, and burst firing.

Intracellular injections of the calcium chelator EGTA

causes DA cells to fire in a regular pacemaker pattern.

Increases in burst firing from a single spiking mode could

be elicited by intracellular calcium injections, and

prevented by intracellular injections of EGTA, suggesting a

calcium involvement in bursting spike patterns (28).

Freeman, et al. (20) studied firing properties of

substantia nigra dopaminergic neurons in freely moving rats. Putative DA neurons were inhibited by injections of apomorphine, and excited by haloperidol. The inhibitions and excitations were associated with a reduction and elevation, respectively, in burst firing.

The ratio of non-bursting to bursting cells indicates that the septal-lesioned group has a much greater proportion of bursting cells than the other groups, with the only exception being the control group. It was suspected that the greater number of bursting cells might account for the increased value of spontaneous firing rates associated with the septal lesioned group. However, as Table II clearly indicates, the bursting cells of the medial septal group did account for the difference in firing rates, but on closer examination, it was the increased firing rates of the bursting cells in conjunction with the increased firing rates of the non-bursting cells that caused the overall increase in firing rates of the habenular neurons of the septal lesioned group.

There is no clear-cut evidence to state that either non-bursting or bursting cells are responsible for the increase in spontaneous activiy of medial habenular neurons of septal animals. The increase in spontaneous firing rates of septal animals occurred in both bursting and non-bursting cell types when compared with three of the experimental groups. When compared with the control group, only the non-bursting cells accounted for a statistically

significant increase in firing rates of septal animals.
The bursting cells of the septal lesioned group were
responsible for the significant difference when this group
was compared with the Sham + 6-OHDA group. The increase
in spontaneous firing rates of the septal group was
attributable to the fact that both bursting and non-
bursting cells fired faster. The emergence of one cell
type being responsible for the faster firing rates became
noticeable only when the septal-lesioned group was compared
to the control or the Sham + 6-OHDA group.

The sympathohabenular ingrowth is considered to be of
a functional nature, but of what kind, inhibitory or
excitatory? It is established that the nature of sympatho-
hippocampal ingrowth is an inhibitory one, but the sympatho-
habenular ingrowth appears to function differently. At the
present time, it has not been determined that this type of
ingrowth is of an inhibitory nature.

There are three reasons supporting this idea. First,
a suggestion was made in the proposal for this dissertation
that increased firing rates might be due to central
noradrenergic fibers which sprout to innervate the medial
habenulae in response to cholinergic denervation. If this
is true, the increased amount of central hypertrophy
presumably would be visible in the medial habenular nuclei
after a septal lesion. A histofluorescence method for
catecholamines was used to determine if the central fibers
were present (17). The group of animals used for the

determination was the MS + BG group. The septal lesion would insure cholinergic denervation, and the bilateral ganglionectomy would insure that no peripheral fibers were present, thus obscuring the vision of central innervation. The rationale was that if central innervation was present and exerting an inhibitory influence, the combined effect of peripheral innervation (if also inhibitory) would produce an excitatory effect, and increased spontaneous firing rates would be observed. Central fibers were not seen in the medial habenulae when selected animals from the MS + BG group were processed for histofluorescence. It cannot be stated that hypertrophy of central noradrenergic fibers was involved in the altered activity of the medial habenular nuclei in septal lesioned rats. In addition, it cannot be stated that the increased firing rates are the result of two inhibitory influences from separate sources serving to cancel each other out, and thus produce an opposite effect.

The second reason also relates to a suggestion made in the dissertation proposal. If the sympathohabenular innervation is acting in an inhibitory fashion, it was originally thought that the injections of 6-OHDA would reverse the inhibitory control to higher than normal baseline firing rates. However, after examining this suggestion more carefully, it is prudent to point out that the 6-OHDA injections would not reverse the nature of firing from sympathohabenular neurons (from inhibitory to

excitatory), but would destroy those neurons such that no firing occurred at all. The Chi-square values obtained from the Kolmogorov-Smirnov test indicate that the groups of animals receiving 6-OHDA injections displayed firing rates that were not significantly different from firing rates of control animals, but were significantly lower than firing rates of septal lesioned animals. This would be due to the decreased number of cells firing, not a change in the nature of the firing cells.

A third observation lending support to the idea that sympathohabenular innervation is not inhibitory is the comparison of firing rates between the MS and the MS + BG groups. If the innervation was inhibitory, it would be expected that the firing rates of the septal lesioned group would be lower than those of the septal lesion + ganglionectomy group. Instead, they were found to be higher, thus suggesting that the sympathohabenular ingrowth is of an excitatory nature.

In summary, it is suggested that sympathohabenular ingrowth is not inhibitory, but perhaps is excitatory, because animals with ingrowth (MS group) had significantly higher firing rates than those observed in the other experimental groups, and the control group. The dissertation did not confirm, but only suggests that the ingrowth is of an excitatory nature.

The dissertation did confirm that increased central noradrenergic fibers were not present in the medial

habenulae after septal lesions. It did not determine if central fibers of other neurotransmitter origins were present, such as fibers of a serotonin, GABA or substance P origin. Further studies should examine the possibility, however remote, that fibers of other transmitter origins innervate the medial habenulae after cholinergic denervation. It also repeated the earlier sympatho-habenular histological results and Crutcher's work (11), in that no peripheral sympathetic innervation was found in the medial habenulae of the normal animal.

There are two further experiments that should be conducted to confirm the suggestion that sympathohabenular ingrowth is excitatory. First, following medial septal lesions and the resulting cholinergic denervation, the possibility exists that synaptic contacts are made by sympathetic terminals onto sites formerly occupied by cholinergic terminals, thus involving a change in postsynaptic receptors. Even though the dissertation did not address this particular issue, an attempt should be made to demonstrate direct postsynaptic effects of electrical stimulation of superior cervical ganglia in the medial habenulae.

Another experiment that would confirm the suggested excitatory nature of sympathohabenular input is one in which norepinephrine, or a noradrenergic analog is directly injected into the medial habenulae after cholinergic denervation. The group of animals to be used would be the

MS + BG group. The medial septal lesion would cause cholinergic denervation, and peripheral sympathetic innervation would be removed after the bilateral ganglionectomy. If the injected norepinephrine caused an increase in firing rates of these animals, it could be stated with further support that sympathetic noradrenergic ingrowth to the medial habenular nuclei is of an excitatory nature.

In conclusion, there are two major results of the dissertation concerning the electrophysiological aspect of the study. First, the disproportionate relationship of non-bursting and bursting cells among the experimental groups and the control group strongly indicated an interesting pattern. As central and peripheral norepinephrine was depleted, the number of non-bursting cells increased.

Second, the firing rates of spontaneous activity of medial septal lesioned animals were significantly greater than the firing rates of animals in all other groups. This high level of mean firing rates is related to a suspected excitatory nature of sympathohabenular ingrowth after septal lesions.

The histological results revealed two notable findings. First, no central noradrenergic innervation was seen in the medial habenulae before or after septal lesions, indicating that the central innervation to the medial habenulae did not hypertrophy in response to cholinergic

denervation. Second, no peripheral sympathetic innervation to the medial habenulae was observed in the normal animal. This finding agrees with one study (11), but not with another group of researchers (3,4,65), who demonstrated sympathetic innervation to the medial habenulae. There is one factor that might explain this difference, and that is the sex of the animals used in the studies. Female rats were used by the researchers demonstrating peripheral sympathohabenular innervation in the normal animal; male rats were used by the researchers who did not demonstrate the normal innervation. On the surface, this appears to be a simplified explanation, but Loy and Milner (41) published a paper indicating sexual dimorphism in the extent of axonal sprouting in the rat hippocampus. They found that after septal lesions in adult males, even though sympathetic ingrowth was elicited, the number of anomalous axons was greatly reduced when compared with adult females. They concluded that the sprouting was affected by the hormonal environment of the hippocampus, or that the hippocampus might possess permanent morphological or physiological differences as a result of exposure to sex steroids during development. Even though this article dealt with post-lesion ingrowth (not pre-lesion sympathetic innervation) to the hippocampus, the possibility does exist that the sex of the animals may be responsible for the presence or absence of sympathetic axons in the normal medial habenulae.

Further studies to be conducted are those which seek

(1) to elicit the mechanism of change in the ratio of non-bursting and bursting cells in response to noradrenergic depletion; (2) to further substantiate the claim that sympathohabenular ingrowth is of an excitatory nature; and (3) to use female rats to determine if normal sympathohabenular innervation can be demonstrated.

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